

# **A Mouse Model for the Overexpression of Methyltransferases**

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*For Sandy, Gerd*

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## Abbreviations and Units

### Abbreviations

5-mC	5-Methylcytosine
A	Adenine
APC	Adenomatosis Polyposis Coli
APS	Ammoniumpersulfate
AS	Angelman Syndrome
BAC	Bacterial Artificial Chromosome
BHMT	Betaine Homocysteine Methyltransferase
BSA	Bovine Serum Albumin
BWS	Beckwith-Wiedemann syndrome
C	Cytosine
C-terminal	Carboxy-terminal
CAG	CMV early enhancer/chicken $\beta$ -actin
cDNA	Complementary DNA
CFP	Cyan Fluorescent Protein
CHD	Choline dehydrogenase
CMV	Cytomegalovirus
CO <sub>2</sub>	Carbon dioxide
CpG	Dinucleotide with the base sequence CG in 5'-3' orientation
Cre	cyclization recombination
CTCF	CCCTC-binding factor
DMD	Differentially Methylated Domain
DMEM	Dulbecco's Modified Eagle's Medium
DMR	Differentially Methylated Region
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DNMT	DNA-methyltransferase
Dnmt1	DNA Methyltransferase 1
Dnmt1o	Oocyte specific isoform of DNA Methyltransferase 1
Dnmt1p	Pachytene sperm specific isoform of DNA Methyltransferase 1
Dnmt1s	Somatic isoform of DNA Methyltransferase 1
Dnmt2	DNA Methyltransferase 2
Dnmt3	DNA Methyltransferase 3
dNTP	Deoxyribonucleotidetriphosphate
dsDNA	Double strand DNA
DTT	Dithiotreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
ES cells	Embryonic stem cells
EtBr	Ethidium bromide
FCS	Fetal Calf Serum
Fig	Figure
FSHD	Facioscapulohumeral muscular dystrophy
FXS	Fragile X-syndrome
G	Guanine
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GNF	Genomics Institute of the Novartis Research Foundation
H <sub>2</sub> O	Water



H3K9	Lysine residue 9 on Histone H3
H3K27	Lysine residue 27 on Histone H3
HA	Hemagglutinin
HAc	Acetic Acid
HCl	Hydrochloric acid
HDAC	Histone deacetylases
HMT	Histone methyltransferase
IAP	intracisternal A particle
IC	Imprinting Center
ICF syndrome	Immunodeficiency, Centromere instability and Facial anomalies syndrome
ID	Imprinting Defect
IFZ	<i>"Institut für Zellbiologie"</i> , Institute for cell biology
Igf2	Insulin-like growth factor 2
IVF	<i>in vitro</i> fertilization
KOH	Potassium hydroxide
LB	Luria Broth
<i>loxP</i>	locus of X-over P1
MAT	methionine adenosyltransferase
mRNA	messenger RNA
MTHF	5-methyltetrahydrofolate
MTHFHM	5-methyltetrahydrofolate-homocysteine methyltransferase
NaOH	Sodium hydroxide
mRNA	Messenger RNA
N-terminal	Amino-terminal
NaOH	Sodium hydroxide
OD	Optical Density
ORF	Open Reading Frame
p	<i>petit</i> ; short arm of a chromosome
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEMT	Phosphatidylethanolamine methyltransferase
PRMT	Protein arginine methyltransferase
PWS	Prader-Willi Syndrome
q	<i>queue</i> ; long arm of a chromosome
RB	Retinoblastoma
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-L-methionine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
<i>Sfrp</i>	secreted frizzled-related protein
SLE	Systemic lupus erythematosus
SV40	Simian Virus 40
SRO	Smallest Region of deletion Overlap
SSC	Saline-Sodium Citrate
T	Thymine
TAE	Tris-actetate-EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine

THF	Tetrahydrofolate
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultra Violet
VT	Versene/Trypsine
ZTL	<i>"Zentrales Tierlaboratorium"</i> , Central Animal Facility

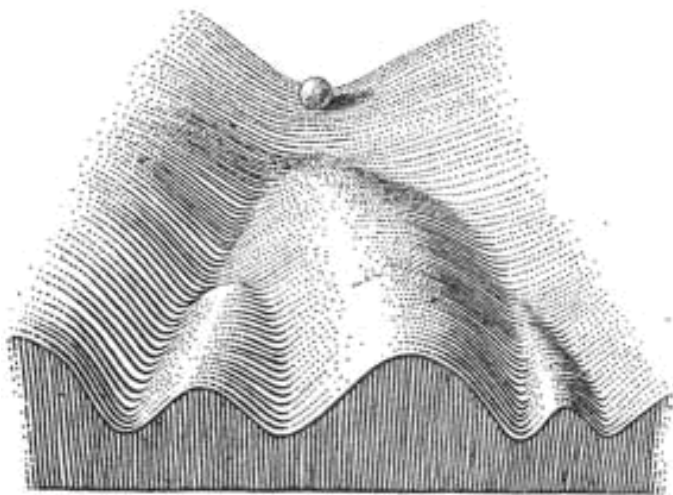
## Units

aa	amino acid(s)
bp	base pair(s)
Bq	becquerel
Ci	curie
°C	degrees celsius
g	gramm(s)
h	hour(s)
J	joule
kb	kilobase(s)
kDa	kilodalton
l	litre
M	molar (mol/l)
μ	micro ( $10^{-6}$ )
m	milli ( $10^{-3}$ )
mA	milliampere
min	minute(s)
ml	millilitre
n	nano ( $10^{-9}$ )
n x g	n-fold earth's acceleration
nm	nanometer(s)
pH	$-\log[H^+]$
rpm	rotations per minute
s	seconds
U	unit(s)
V	volt
v/v	volume per volume
w/v	weight per volume

## 1. Introduction

### 1.1 The Epigenetic Regulation of Genes

The term “epigenetics” was coined over 60 years ago by Conrad Hal Waddington when he combined the words “epigenesis” and “genetics”. He realized that “the fertilized egg contains some preformed elements – namely, genes and a certain number of different regions of the cytoplasm – and (...) that during development these interact in epigenetic processes to produce the final adult characters and features that are not individually represented in the egg” (Waddington, 1957; Waddington, 1966). By representing a developmental pathway as a valley in a landscape, Waddington provided a simple mechanical analogy for the rather complex epigenetic mechanisms that occur in organisms during development (Fig.1). An important part of Waddington’s definition is the incorporation of cytoplasmic elements in addition to nuclear elements, which allows for the influence of extrinsic factors, such as nutrition, on gene regulation. This idea has often been underrated in more recent definitions where epigenetics has been described, for example, as “all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself” (Egger *et al.*, 2004).

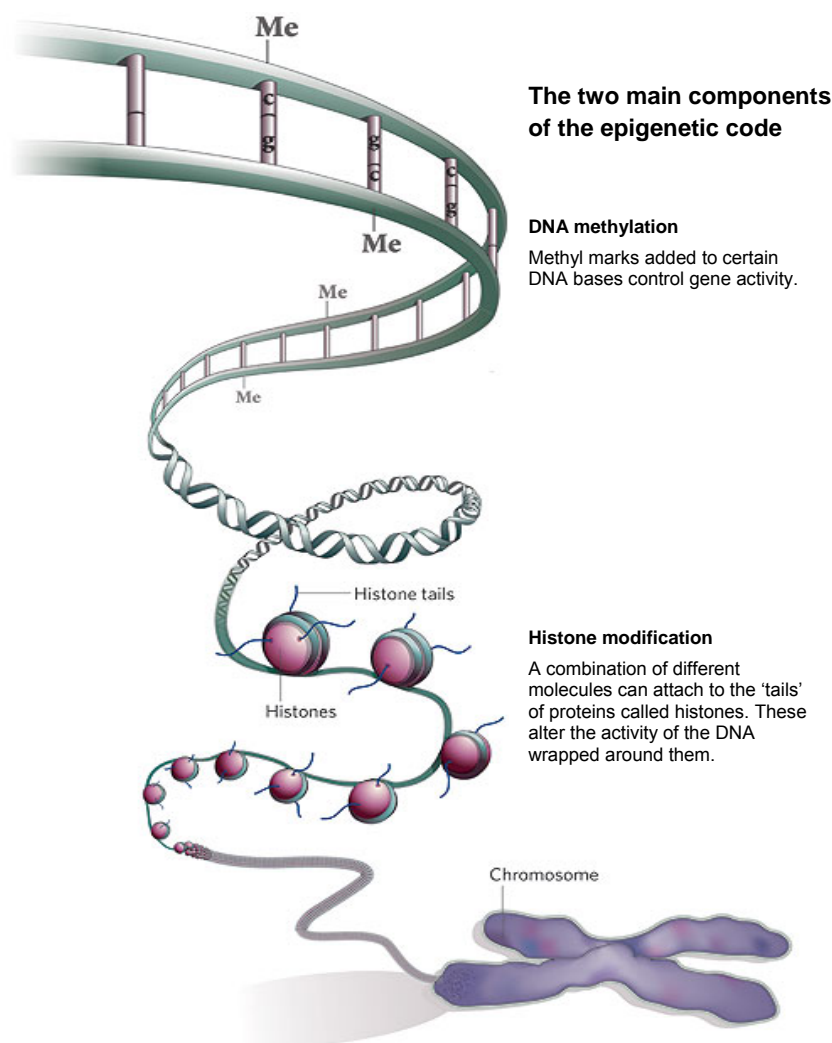


**Figure 1. The epigenetic landscape**

A number of possible pathways of development lie ahead of the cells of a developing embryo, and at any particular point in an embryo’s development these potential pathways can be switched. The developmental pathways that could be taken by each embryo cell are metaphorically represented by the pathway taken by the ball as it rolls down the valleys. The pathways can be influenced either by genetic factors, or by developmental

plasticity (the ability to respond to environmental factors). Up to a certain threshold, any genetic variation or environmental noise will be “buffered” and will not affect the canalization, but above this threshold, the cell (ball) would flip over into an adjacent pathway. (Figure adapted from Waddington, 1957 and Slack 2002; © (1957) Geo Allen & Unwin)

The transcription of genes is regulated in *cis* by recognition sequences for transcription factors (promoter, silencer, insulator and enhancer elements) and in *trans* by transcription factors that bind to the *cis*-regulative elements (Alberts *et al.*, 2002). In addition to the direct mechanisms which control gene expression, like the binding of activators or repressors to promoter or enhancer sequences, there are epigenetic modifications that control transcription by influencing the chromatin structure without changing the underlying base sequence. In this way they may control the accessibility of binding sites to transcription factors. Epigenetic modifications are cell-heritable, and can thereby lock in gene expression states throughout development and reproduction. Research has focused on DNA-methylation and histone modifications (Fig. 2). They are often referred to as the two main mechanisms of modern molecular epigenetics that deal with the variation and inheritance of states of gene expression (Horsthemke, 2005a).



**Figure 2. The two main components of the epigenetic code**

The figure shows the sites of DNA-methylation and histone modifications in the chromosome. Me: methylated cytosine base. (Figure adapted from Qiu, 2006)

DNA-methylation is involved in the epigenetic regulation of gene expression, as certain gene regions allow the binding of transcription factors only if they contain the correct specific methylation pattern (Vanyusin, 2004). Histone modifications are the second important factor for epigenetic regulation of gene expression, as they can directly influence chromatin structure, providing a steric mechanism of control for transcription factor binding. Furthermore, the two hallmark mechanisms of epigenetics are closely connected and even interact in some cases (Fuks, 2005; Estève *et al.*, 2006).

## 1.2 DNA Methylation

Pioneer studies in DNA methylation date back over 50 years, when it was first discovered that methylated bases can occur in DNA along with the four classical bases (adenine, guanine, cytosine, and thymine). 5-Methylcytosine (5-mC) was first found in various types of DNA by Hotchkiss (1948) and Wyatt (1950), although its synthesis and function remained unclear for some time. In 1963, the DNA-methyltransferases (DNMTs) were observed for the first time (Gold and Hurwitz, 1964). These are specific enzymes that transfer methyl groups from the donor molecule S-adenosyl-L-methionine (SAM) selectively onto cytosine residues in eukaryotic DNA chains. It became clear that methylated bases are not incorporated into eukaryotic DNA in a ready-made form, but originate from enzymatic methylation of cytosine in DNA chains that are forming or already formed.

In eukaryotic DNA, methylated cytosines are formed mainly in CpG sequences, the regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases (Vanyushin, 2004). In mammals about 70 % of CpG cytosines are methylated (Robertson and Jones, 2000). However, the methylated fraction of the human genome is rather small (about 4 % of the cytosines are methylated) and the methylated cytosines are distributed in complex patterns over the genome (Lyko, 2005). Regions of DNA of greater than 500 bp with a GC content equal to or greater than 55% and a ratio of observed CpG/expected CpG of 0.65 or more are known as CpG islands (Takai and Jones, 2002). About half of all known genes in mammalian genomes have CpG islands associated with the start of the

gene (Jabbari and Bernardi, 2004). The presence of CpG islands is therefore used to help in the prediction and annotation of genes (Bird, 1987; Gardiner-Garden and Frommer, 1987). Although the aforementioned context in which methylated cytosines occur has been widely accepted over the past years, a study published in 2009 that compared the methylome of human embryonic fibroblasts to that of fetal lung fibroblasts has now confirmed the finding of two previous studies that the 5-mC does not only occur in a CpG context in mammalian cells (Woodcock *et al.*, 1987; Ramsahoye *et al.*, 2000; Lister *et al.*, 2009). This study showed that nearly one quarter of all 5-mC residues identified in embryonic stem cells were not in a CpG context, suggesting that embryonic stem cells may use different methylation mechanisms than somatic cells to affect gene regulation (Lister *et al.*, 2009).

Further studies found that methylation of cytosine residues plays a vital role in stabilizing the double helix, inactivation of the X-chromosome, differential inactivation of genes during embryogenesis, and genetic imprinting (Vanyushin *et al.*, 1970; Riggs, 1975; Li, 1992; Bell and Felsenfeld, 2000). In many cases, cytosine methylation prohibits the binding of specific nuclear proteins involved in transcription, replication, and DNA repair (Bird and Wolffe, 1999). Moreover, many types of cancer use methylation mechanisms to gain control over the regulation of certain genes (Baylin *et al.*, 1986; Greger *et al.*, 1989; Herman and Baylin, 2003; Feinberg and Tycko, 2004).

### **1.2.1 Gene Regulation by DNA Methylation**

First observations that 5-mC plays a role in the regulation of various eukaryotic genes were made in 1977 by the inhibition of DNMTs (Constantinides *et al.*, 1977). Later studies showed that the methylation of CpG dinucleotides in promoter regions can control gene expression (Sutter and Doerfler, 1980; Vardimon *et al.*, 1980; Langner *et al.*, 1984; Arney, 2003), mostly by binding co-repressors called methyl-CpG-binding proteins (MeCPs). These co-repressors can in turn recruit histone modifying enzymes, such as the histone deacetylases (HDACs), to the promoter region, which then sterically modify the chromatin structure to prevent gene expression (Bird and Wolffe, 1999; Jaenisch and Bird, 2003).

The methylation of DNA has specifically been shown to influence the epigenetic regulation of imprinted genes (Delaval and Feil, 2004), such as Insulin-like growth factor 2 (*Igf2*), which are expressed either from the paternal or from the maternal allele. This differential expression is regulated, among others, by DNA-methylation. As one allele is methylated, and the other is unmethylated, only one of the alleles is genetically expressed. In most cases, the unmethylated allele is expressed while the methylated allele is silenced.

To date there are more than fifty known human imprinted genes ([http://www.har.mrc.ac.uk/research/genomic\\_imprinting/](http://www.har.mrc.ac.uk/research/genomic_imprinting/), <http://www.geneimprint.com/site/genes-by-species>). Although there are no known common functional domains or protein sequences among imprinted genes, they are often clustered in certain chromosomal regions (Reik and Maher, 1997; Reik and Walter, 2001), indicating that the establishment and maintenance of imprinted genes may be regulated coordinately in these regions, referred to as imprinting centers (ICs).

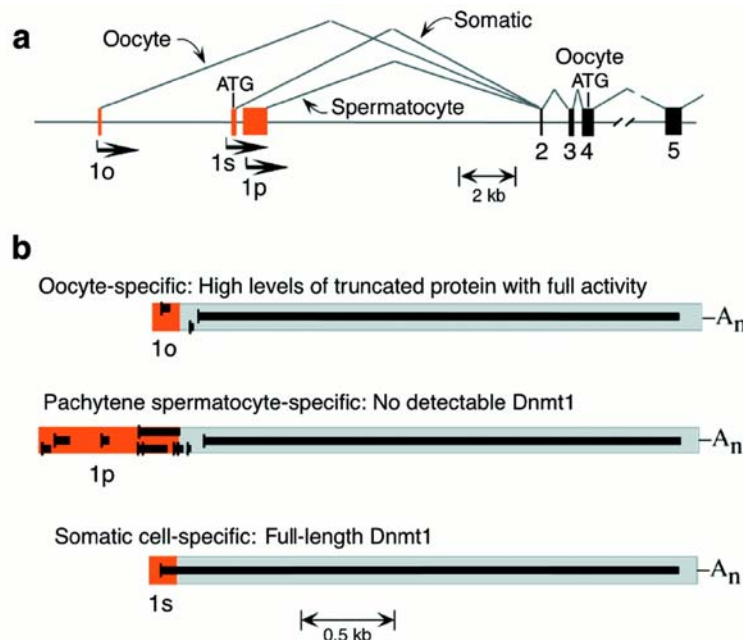
In contrast to the mechanisms of most known imprinted genes, where hypermethylation of promoter regions leads to gene silencing, the expression of *Igf2* is increased by the hypermethylation of certain CpG islands in the *Igf2/H19* complex, whereas their hypomethylation leads to gene silencing. Various regions of the *Igf2/H19* complex contain CpG islands which are regulated by the change of their methylation status (Arney, 2003). One of these regions, known as differentially methylated region 2 (DMR2), lies 3' of the promoter region of *Igf2*. Methylation of DMR2 prevents binding of the repressor GC-binding factor 2 (GCF2), resulting in expression of *Igf2* (Bell and Felsenfeld, 2000). Another part of the *Igf2/H19* complex that is influenced by methylation is the *H19* differentially methylated domain (DMD), which lies 5' of the *H19* gene region. Methylation of the DMD prevents binding of an insulator protein, the zinc-finger protein CCCTC-binding factor (CTCF), thus allowing interaction of the *Igf2* promoter with enhancers downstream of *H19*, so that *Igf2* is upregulated (Arney, 2003).

### 1.2.2 DNA Methyltransferases

There are two basic categories of DNMTs, “*de novo*” and “*maintenance*” DNA-methyltransferases. The maintenance methyltransferase Dnmt1 prefers to methylate newly synthesized CpGs whose partners on the parental strand are already methylated (Bestor, 1992; Pradhan *et al.*, 1999). As 5-mC is not available as a free base for incorporation during semiconservative replication, the newly synthesized DNA strand *per se* does not contain any methylation marks, resulting in hemi-methylated DNA. The CpGs of the daughter strand are modified according to the original helix, preserving the methylation pattern during semiconservative replication (Holliday and Pugh, 1975; Riggs, 1975; Bird, 2002). Since 1995, various studies have also attributed *de novo* activity to Dnmt1 (Christman *et al.*, 1995; Tollefsbol and Hutchison, 1995; Yoder *et al.*, 1997), which until then had only been proven to function as a maintenance methyltransferase. One study even attributes a higher specific activity of Dnmt1 on unmethylated substrates than that of Dnmt3a and Dnmt3b (Okano *et al.*, 1998). Interestingly, the preferential recognition sequence for Dnmt1s *de novo* methylation is CCGG, which is disfavoured by Dnmt3a and Dnmt3b (Goyal *et al.*, 2006).

The *Dnmt1* gene is located on chromosome 9 in the mouse and has three mRNA isoforms, known as *Dnmt1o*, *Dnmt1p* and *Dnmt1s*, that differ in alternative first exons (Fig. 3). *Dnmt1o* and *Dnmt1p* are sex-specific isoforms that are only expressed in the oocyte up to the 8-cell stage and in the pachytene spermatocyte, whereas *Dnmt1s* is the somatic form that is expressed in the oocyte from the 2-cell stage to the adult cell stage (Mertineit *et al.*, 1998; Cirio *et al.*, 2008a).





**Figure 3. Sex-specific exons and mRNAs from the *Dnmt1* gene**

(a) 5' region of *Dnmt1* on proximal mouse chromosome 9: exon 1o is oocyte-specific; exon 1s is specific to somatic cells of both sexes; and exon 1p is restricted to pachytene spermatocytes. The ATG codon in exon 1s is used for initiation of translation in somatic cells; a truncated form arises from use of the ATG codon in exon 4 in oocytes.

(b) mRNA products of sex-specific exons. Effect of the alternative promoter use and splicing on organization of mature *Dnmt1* mRNAs are indicated. Heavy horizontal bars indicate open reading frames; short vertical bars indicate ATG initiation codons. (Figure from Bestor, 2000)

*Dnmt1o* expression is driven by a promoter upstream of the *Dnmt1s* promoter, which introduces an untranslated exon (exon 1o) that shifts translation initiation to a start codon in exon 4 of *Dnmt1*. This results in a truncated form of *Dnmt1s* that lacks 118 N-terminal amino acids. Interestingly, *Dnmt1o* shows low levels of mRNA expression, but high levels of enzymatically active protein in the oocyte, and furthermore has the ability to travel from the cytoplasm to the nucleus and back (Carlson *et al.*, 1992; Mertineit *et al.*, 1998).

*Dnmt1p* has the same open reading frame as *Dnmt1o*, but is expressed from a promoter which lies downstream of the *Dnmt1s* promoter. In contrast to *Dnmt1o*, *Dnmt1p* shows high mRNA levels in the pachytene sperm, but so far no corresponding protein has been detected. This may be caused by interference of multiple open reading frames contained in Exon 1p, which may interfere with *Dnmt1p* expression (Trasler *et al.*, 1992; Mertineit *et al.*, 1998).

*Dnmt1s* is the ubiquitously expressed somatic form, and is the only isoform expressed later than the 8-cell stage of the oocyte in human and murine adult somatic cells (Yoder *et al.*, 1996). *Dnmt1s* is a large enzyme of 1620 aa that can be subdivided into an N- and a C-terminal domain (Jeltsch, 2006). It targets replication

foci using recognition sequences at the N-terminal end, which contains several individual domains for activity regulation and intracellular targeting (Gruenbaum *et al.*, 1983; Chuang *et al.*, 1997; Leonhardt *et al.*, 1992; Jeltsch, 2006). The C-terminal end contains the catalytic center and shares sequence motif homology with prokaryotic cytosine-5 methyltransferases (Jeltsch, 2002). In contrast, there is only little homology between Dnmt1 and Dnmt3a/3b (Okano and Li, 2002).

*Dnmt2* is a well-conserved gene with orthologs in plants, yeast, *Drosophila*, humans, and mice. Although the motifs essential for methylation activity are present, the catalytic activity of Dnmt2 in mammals is still a controversial issue (Liu *et al.*, 2003).

The *de novo* DNMTs Dnmt3a and Dnmt3b introduce methylation marks into DNA helices that are not methylated on either strand (Okano *et al.*, 1999; Goll and Bestor, 2005). *De novo* DNMT activity is of special importance during the early stages of embryogenesis, after DNA methylation marks on the paternal allele have been erased actively (Mayer *et al.*, 2000; Oswald *et al.*, 2000) and some regions of the maternal allele are subjected to passive demethylation (Rougier *et al.*, 1998). At the blastula stage the genome is remethylated, and this is one of the main functions performed by the *de novo* methyltransferases. Dnmt3a and Dnmt3b have a 13-fold higher affinity for the flanking base-pairs RCGY than for YCGR (R = A or G and Y = C or T), and also prefer AT-rich flanks over GC-rich ones (Handa and Jeltsch, 2005). Dnmt3b has one enzymatically active isoform, Dnmt3b1, and two enzymatically inactive truncated isoforms that are produced by alternative splicing, namely Dnmt3b3 and Dnmt3b6 (Chen *et al.*, 2003). The establishment and maintenance of DNA methylation patterns in mammals has been shown to be coordinated cooperatively by the Dnmt1 and Dnmt3 families (Lyko *et al.*, 1999; Okano *et al.*, 1999; Kim *et al.*, 2002; Chen and Li, 2006).

### 1.3 Histone Modifications

In the eukaryotic cell nucleus, DNA is not present in a free form, but is complexed to chromatin by its association with core histones and other non-histone chromosomal proteins. Partially unfolded chromatin has a “beads on a string” structure (Fig. 2). The “string” is the DNA strand, and the “beads” are made up of the DNA “string” wrapped

around a histone protein core. The histone protein core consists of two molecules each of histones H2A, H2B, H3 and H4; the double stranded DNA wrapped around it is 146 bp long. This “bead” structure is referred to as the “nucleosome core particle”. Between the nucleosome core particles are regions of linker DNA (the “string”) of up to about 80 bp (Alberts *et al.*, 2002).

The histone core has a globular C-terminal region that connects the histones and binds to the DNA, and a linear N-terminal region of 20 – 36 amino acids which can be targeted by various types of posttranslational modifications (Fig. 4). Among these modifications are the acetylation and methylation of lysine and arginine, the phosphorylation of serine and threonine, the sumoylation and ubiquitylation of lysine (Spivakov and Fisher, 2007). Several types of covalent modification, such as acetylation and lysine methylation, are reversible. These modifications can directly influence the condensation and structure of chromatin and thereby influence the transcription of genes.

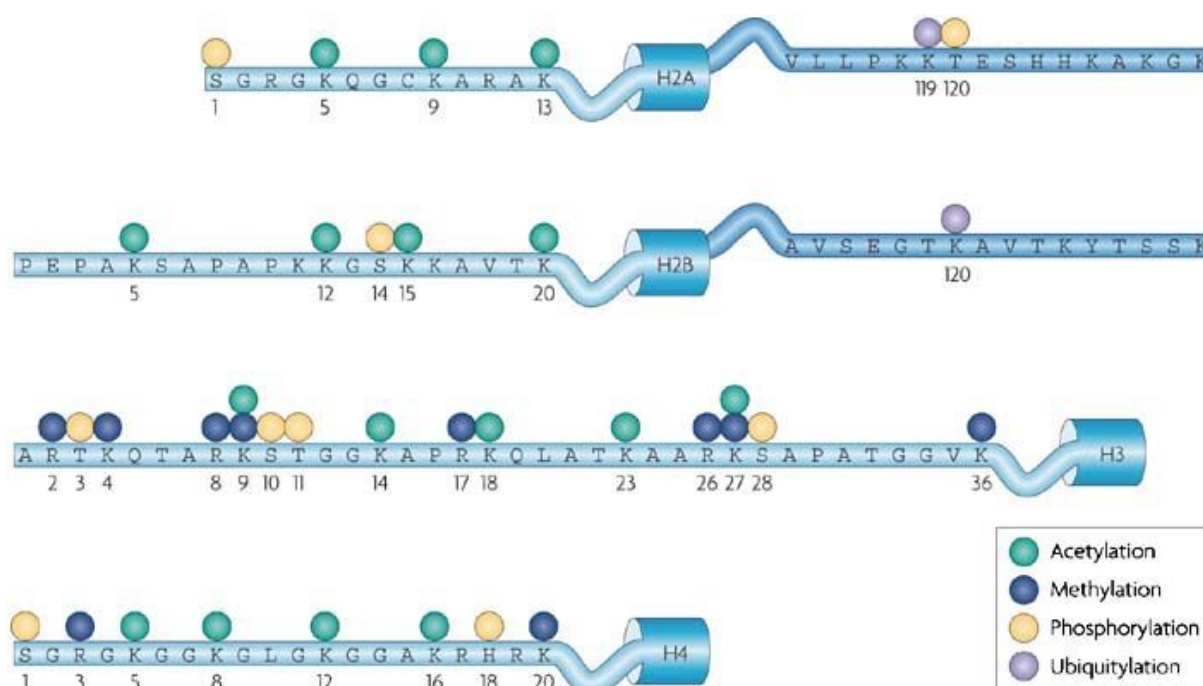
### **1.3.1 Gene Regulation by Histone Modifications**

Histone acetylation was the first modification that was shown to regulate gene expression by affecting the dynamics of chromatin structure (Brownell *et al.*, 1996). In 1999, first evidence that histone methylation can also regulate gene expression was presented (Chen *et al.*, 1999). Interestingly, the modification of a specific residue can lead to both gene activation and gene inactivation. For example, the acetylation of lysine residue 9 on Histone H3 (H3K9) is typically found in chromatin that is transcriptionally active, whereas H3K9 methylation is associated with a transcriptionally inactive state. Furthermore, specific types of modification do not consistently indicate activation or inactivation. The methylation of lysine 4 on histone H3 (H3K4), for example, is a marker for an active state of transcription. The methylation of lysine 27 on the same histone (H3K27), however, is a marker for an inactive state of transcription. Consequently, histone modifications are a versatile mechanism for eukaryotic cells to regulate gene expression.

### 1.3.2 Histone Methyltransferases

Histone methyltransferases (HMTs) are categorized into two families: Protein arginine methyltransferases (PRMTs), which catalyze the transfer of methyl groups from SAM to arginine residues, and the SET domain family of HMTs, which are able to methylate lysine residues (Zhang and Reinberg, 2001).

The three most prominent members of the SET domain family of HMTs are Suv39h1, Suv39h2 and G9a. The first two were identified by Jenuwein and colleagues as lysine-preferring mammalian HMTases in 2000, respectively (Rea *et al.*, 2000; O'Carroll *et al.*, 2000). Both were shown to selectively methylate H3K9. G9a, discovered by Tachibana and colleagues in 2001, also transfers methyl groups to H3K9, but with 10 to 20-fold higher activity than Suv39h1. Additionally, it can methylate lysine 27 of histone H3 (H3K27) (Tachibana *et al.*, 2001).



**Figure 4. Posttranslational histone modifications**

Posttranslational histone modifications mostly take place on the N-terminal tails of the core histones H2A, H2B, H3 and H4. Here the known sites of possible acetylation, methylation, phosphorylation and ubiquitylation are shown. Some amino acids can be targeted by more than one type of modification. (Figure from Spivakov and Fisher, 2007)

## 1.4 Diseases Resulting from Epimutations

Errors in the establishment or maintenance of DNA-methylation patterns and histone-modification patterns can wrongly lead to the activation of a silent gene or the inactivation of an active gene (Horsthemke, 2005b). Epimutations are split into two groups, primary and secondary epimutations, and comprise these types of errors (Holliday, 1987).

Primary epimutations are most likely to be the consequence of stochastically occurring errors in the composition or maintenance of the epigenetic status of a gene without change of the DNA sequence itself. They can occur during the deletion of genomic imprints in primordial germ cells, during the subsequent re-establishment of imprints, or after fertilization during the maintenance of imprints. The rate of spontaneous primary epimutations can be influenced by intrinsic (genetic) or extrinsic factors (Horsthemke, 2005b).

Secondary epimutations are the consequence of mutations in the DNA sequence and can thus be *de novo* or inherited. They can influence gene expression in *cis* when they are in or close to an affected gene, or in *trans* when they affect the function of a protein that controls the gene's epigenetic state. Secondary epimutations are known to be the cause for at least two diseases, the Fragile X-syndrome (FXS), caused by a mutation in the *FMR1* gene (De Boulle *et al.*, 1993), and Facioscapulohumeral muscular dystrophy (FSHD), where the copy number of the variable number tandem repeat D4Z4 is reduced to below 11 by deletion, in comparison to 11 – 150 copies in healthy individuals (Lunt *et al.*, 1995; Lunt, 1998).

Some types of disease have been shown to be associated with both primary and secondary epimutations. Among these diseases are various types of cancer and imprinting defects such as the Prader-Willi syndrome (PWS), the Angelman syndrome (AS) and the Beckwith-Wiedemann syndrome (BWS) (Buiting *et al.*, 1995; Buiting *et al.*, 2003; Walter and Paulsen, 2005). Furthermore, some diseases are caused by defects in the methylation machinery itself, such as the Immunodeficiency, Centromere instability and Facial anomalies syndrome (ICF syndrome), caused by a mutation in the *Dnmt3b* gene (Hansen *et al.*, 1999; Jiang *et al.*, 2005), and Systemic lupus erythematosus (SLE, or commonly, “lupus”), which is characterized by DNA-hypomethylation of T lymphocyte DNA (Sekigawa *et al.*, 2003; Sawalha and

Richardson, 2005). The spectrum of diseases confirms the multiple proposed roles of DNA methylation, including transcriptional regulation, chromosomal structure and chromosomal stability.

#### **1.4.1 Imprinting Defects in the Prader-Willi and Angelman Syndromes**

Imprinted genes are usually expressed either from the paternal or the maternal allele, with the other allele remaining silent. Aberrant methylation can change the expression profile of imprinted genes, so that they are expressed from neither or both of the alleles. Recent studies indicate that *in vitro* fertilization (IVF) may be a factor that increases the risk of imprinting defects (Hiendleder *et al.*, 2004).

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are distinct neurogenetic disorders and the first known examples of human diseases involving imprinted genes. In a small subset of patients with PWS (~ 1 %) or AS (~ 2 - 4 %) the disease is caused by an imprinting defect, leading to aberrant methylation and expression of imprinted genes in the chromosomal region 15q11q13. PWS is caused by the loss-of-function of paternally expressed genes in this region and characterized by a mild to moderate mental retardation, short stature, small hands and feet, hypogonadism, hyperphagia, adipositas, sleep apnea and behavioral problems. A loss-of-function of the maternally expressed *UBE3A* gene causes AS, a neurogenetic disease characterized by mental retardation, jerky movements, lack of speech and friendly behavior.

Parent-specific gene expression is regulated by an imprinting centre (IC) within 15q11q13. It consists of two critical elements, the AS-SRO and the PWS-SRO (smallest region of deletion overlap). They are defined by determining the smallest overlapping regions of IC-deletions in PWS and AS patients. The PWS-SRO is responsible for post-zygotic maintenance of the paternal imprint in the early embryo (Bielinska *et al.*, 2000; El-Marri *et al.*, 2001), whereas the AS-SRO possibly interacts with the PWS-SRO to establish the maternal imprint in the female germline (Buiting *et al.*, 1995; Dittrich *et al.*, 1996; Shemer *et al.*, 2000; Perk *et al.*, 2002; Kantor *et al.*, 2004a; Kantor *et al.*, 2004b).

A secondary epimutation in form of a microdeletion of the IC is the cause of imprinting defects in 10 – 15 % of cases (Buiting *et al.*, 1995; Saitoh *et al.*, 1996; Schuffenhauer *et al.*, 1996; Ohta *et al.*, 1999b; Buiting *et al.*, 2000). However, the majority of patients with an imprinting defect (ID), (85 – 90 %), have a primary epimutation without DNA sequence changes (Buiting *et al.*, 2003). This primary epimutation leads to a paternal chromosome carrying a maternal imprint in ID-patients with PWS; in ID-patients with AS the maternal chromosome carries a paternal imprint. In PWS patients the chromosome 15 with a primary epimutation is always inherited from the paternal grandmother (Buiting *et al.*, 2003). This is probably due to a failure to erase the paternal grandmother's imprint during spermatogenesis. In contrast, the chromosome 15 with a primary epimutation in AS patients can be inherited from the maternal grandfather or grandmother, indicating that the erroneous imprinting occurs after fertilization, and thus could be caused by an error either in the establishment, or in the maintenance of the maternal imprint.

#### **1.4.2 DNA Methylation and Cancer**

Alterations in the methylation pattern of DNA can play an important role in the development of tumors, although it is uncertain if DNA methylation alone is sufficient to trigger cancer development (Bestor, 2003; Lyko, 2005; Jones and Baylin, 2007). Tumor cells are generally characterized by global hypomethylation of the genome (Feinberg and Vogelstein, 1983; Ehrlich, 2002), accompanied by region-specific hypermethylation (Baylin *et al.*, 1986; Robertson and Jones, 2000).

Global hypomethylation has been linked to a reduction in genome stability, a hallmark of many cancers, and can occur due to the reduced availability of methyl group donors (Blusztajn, 1998; Lyko, 2005). Global hypomethylation has also been shown to induce tumorigenesis in several different mouse models (Eden *et al.*, 2003; Gaudet *et al.*, 2003; Yamada *et al.*, 2005; Jones and Baylin, 2007).

Apart from a few exceptions (like the aforementioned *Igf2*), region-specific hypermethylation is closely associated with epigenetic gene silencing and therefore effectively results in loss-of-function mutations (Jones and Baylin, 2002; Prawitt and Zabel, 2005).



It has been shown that the promoter region of tumor suppressor genes can become hypermethylated during tumorigenesis (Greger *et al.*, 1989; Herman and Baylin, 2003, Feinberg and Tycko, 2004). Among others, this has been associated with the transcriptional silencing of the retinoblastoma (*RB*) tumor suppressor gene in patients with retinoblastoma tumors (Greger *et al.*, 1994). Further studies have suggested that *de novo* methylation has a causal role in the development of tumors, and that hypermethylation is already detectable in the earliest stages of tumor development (Myohanen *et al.*, 1998; Chan *et al.*, 2006). One *in vitro* study addressing the overexpression of *Dnmt1* in murine cells indicated that hypermethylation may promote cell transformation (Wu *et al.*, 1993), but a further study that transferred this approach into a mouse model had an embryonic lethal phenotype, so that it could not be used to observe the long-term effects of *Dnmt1* overexpression (Biniszkiewicz *et al.*, 2002). Most other studies investigating the role of DNA methylation in cancer examine loss-of-function by inhibition of Dnmt1 (Robert *et al.*, 2003). Therefore, it remains unclear whether DNA methylation is one of the multifactorial trigger factors of cancer development, or if it is induced by cancer development, or both (Bestor, 2003; Lyko, 2005; Jones and Baylin, 2007). Clearly, more gain-of-function studies, preferably *in vivo*, are necessary to elucidate this question.

### **1.5 DNA Hypermethylation by Overexpression of DNA Methyltransferases**





DNA hypermethylation can be triggered by an overexpression of DNA methyltransferases due to cancer (as mentioned in 1.4.2) or genetic modification (Biniszkiewicz *et al.*, 2002; Linhart *et al.*, 2007), but is also due to the dietary availability of methyl group donor nutrients such as folate and choline (Blusztajn, 1998; Kovacheva *et al.*, 2007; Kovacheva *et al.*, 2007). The *in vivo* overexpression of DNMTs was demonstrated for *Dnmt1* (Biniszkiewicz *et al.*, 2002) and for *Dnmt3a* and *Dnmt3b* (Linhart *et al.* 2007). Biniszkiewicz and colleagues established murine embryonic stem (ES) cells that overexpress *Dnmt1* using a bacterial artificial chromosome (BAC) transgene. Their study showed that the cultured cells have an increased methylation of intracisternal A particles (IAPs) and an increased methylation of the maternal *Igf2* allele that is usually unmethylated due to genomic



imprinting. It also showed that the methylation of the maternal allele results in an increase of *Igf2* expression due to a shift from monoallelic to biallelic expression of *Igf2* (Fig. 6). The developmental potency of the transgenic ES cells was investigated by implanting them into tetraploid blastocysts. Because tetraploid blastocysts cannot contribute to embryonic lineages, the composite embryos give rise to mice that are entirely derived from the descendants of ES cells injected into the blastocyst (Nagy *et al.*, 1990; Nagy *et al.*, 1993). The offspring of these blastocyst injections had an embryonic lethal phenotype, which was attributed to the consequences of *Dnmt1* overexpression.

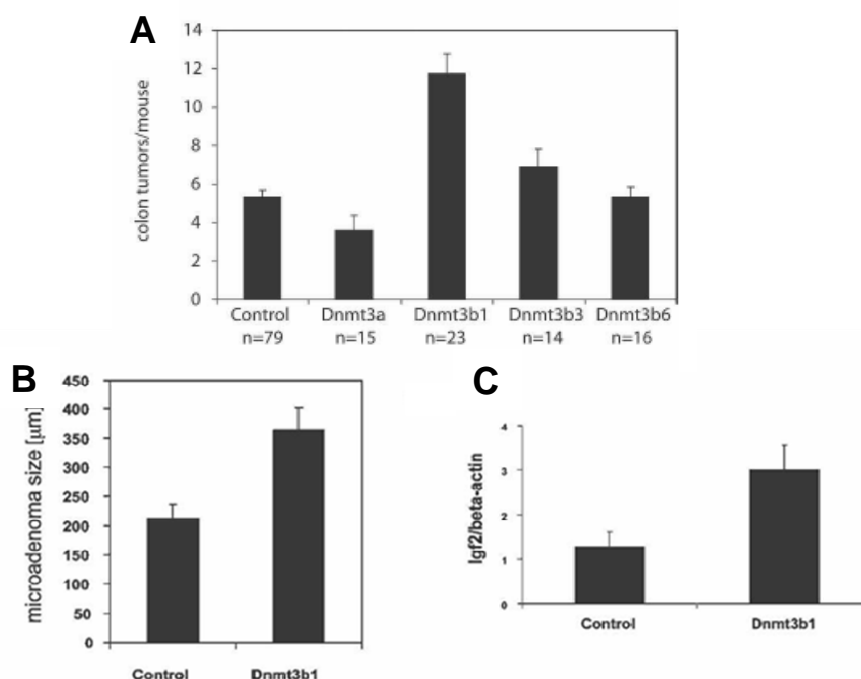
Genotype	Dnmt1	IAP		Igf2r			Igf2			embryonic phenotype		
	expression by Western	methylation	expression	methylation mat.	expression pat.		methylation mat.	expression pat.				
+/+	100%		-			+			+	93%	7%	normal
+/+;BAC	400%		-			+			++	32%	68%	lethal

**Figure 6. Summary of methylation and expression status of repetitive sequences such as IAP and of the imprinted genes *Igf2r* and *Igf2* in cells with different levels of *Dnmt1* expression, as measured by Western blot analysis (shown in second column)**

Repetitive sequences (IAP) and imprinted genes (*Igf2r*, and *Igf2*) are subject to postzygotic *de novo* methylation, as illustrated. Repetitive IAP sequences are highly susceptible to *de novo* methylation. *Igf2r* is completely resistant to *de novo* methylation. The imprinted region of *Igf2* and *H19* becomes fully methylated at a 4-fold level of *Dnmt1* expression. The maternal and paternal alleles of *Igf2* and *Igf2r* are indicated. The expression levels of IAP, *Igf2r*, and *Igf2* are indicated by the number of “+” signs, while a “-” sign indicates no expression. Monoallelic or biallelic *Igf2* expression in differentiated cells is indicated as the percentage of total *Igf2*-expressing cells. Embryonic survival of the ES cell tetraploid blastocyst-derived mice is summarized in the last column. Symbols: , unmethylated; , partially methylated (low); , partially methylated (high); , methylated; oval with one dot, monoallelic *Igf2* expression; oval with two dots, biallelic *Igf2* expression. (Figure adapted from Biniszkiwicz *et al.*, 2002)

Linhart and colleagues established transgenic mouse lines for the overexpression of *Dnmt3a* and *Dnmt3b1* (Linhart *et al.*, 2007). After crossing in the *Dnmt3b1* overexpressing line with APC<sup>Min/+</sup> mice, which are susceptible to colon tumors, they observed an increase in the number and size of intestinal adenomas and microadenomas as well as an increase in *Igf2* expression due to *H19* DMD hypermethylation in comparison to the APC<sup>Min/+</sup> control mice (Fig. 7). In contrast, there were no indications of consequences resulting from the overexpression of *Dnmt3a*, even after inbreeding with APC<sup>Min/+</sup> mice. They also found that the secreted frizzled-related protein (*Sfrp*) genes *Sfrp2*, *Sfrp4* and *Sfrp5* were significantly *de novo* methylated in tumor samples of the *Dnmt3b1* mice.

The *Sfrp* genes are considered as inhibitors of the Wnt pathway, which is a key component of most intestinal tumors (Clevers, 2006). In contrast to *Igf2* they underlie the conservative mechanism for epigenetic regulation, so their methylation results in downregulation of expression, which may have contributed to the increased number of tumors in the *Dnmt3b1* mouse model (Caldwell *et al.*, 2004; Suzuki *et al.*, 2004). Interestingly, *Sfrp2* and *Sfrp5* were also hypermethylated in normal intestinal mucosa, indicating that *de novo* methylation can lead to a silencing of *Sfrp* genes. This finding supports the hypothesis that DNA methylation is a trigger factor rather than a consequence of transcriptional silencing in carcinogenesis (Gu *et al.*, 2006). Taken together, the research on DNMTs in cancer in combination with the publications Biniszkievicz and Linhart shows that the overexpression of DNMTs can trigger DNA hypermethylation, although certain levels of overexpression, or certain predispositions to hypermethylation, may be necessary for the effect to take place.



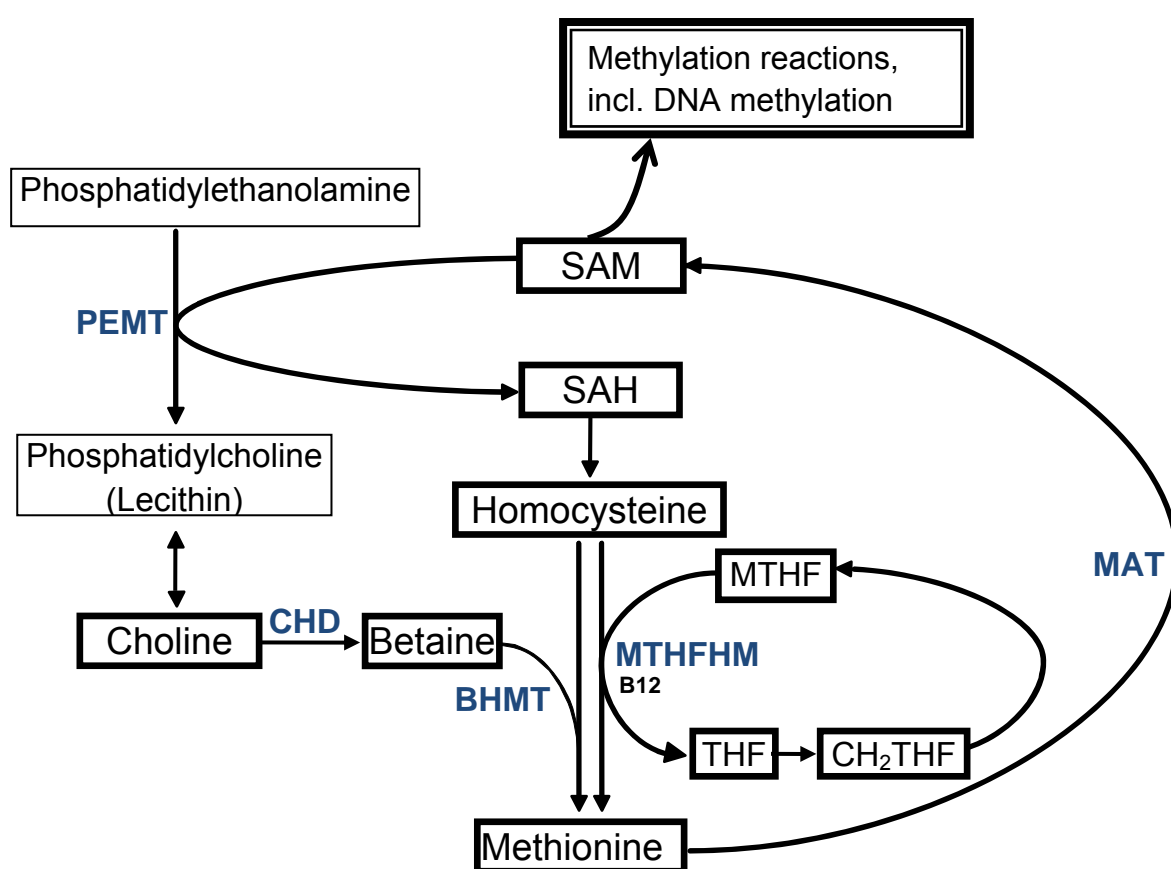
**Figure 7. *Dnmt3b1* overexpression increases the number of intestinal tumors, the size of colonic microadenomas and the expression of *Igf2***

(A) Number of macroscopic colon tumors per mouse. Increased expression of *Dnmt3b1* causes a 2.2-fold increase in the number of colon tumors per mouse ( $11.7 \pm 1$  vs.  $5.3 \pm 0.3$  colon tumors per mouse;  $P < 0.0002$ , Mann-Whitney test). (B) The average size of colonic microadenomas in *Dnmt3b1*-expressing mice increases 1.7-fold when compared with controls ( $364 \mu\text{m} \pm 38$  vs.  $211 \mu\text{m} \pm 25$  colon;  $P < 0.0005$ ). Values represent mean  $\pm$  SE. (C) Quantitation of *Igf2* expression in colon tumors normalizes to  $\beta$ -actin expression using real-time PCR. The relative *Igf2* expression in tumors derived from *Dnmt3b1* mice ( $2.9 \pm 0.6$ ,  $n = 10$ ) is significantly higher than *Igf2* expression in control mice ( $1.3 \pm 0.4$ ,  $n = 11$ ),  $P < 0.03$  Mann Whitney U-test. (Figure from Linhart *et al.*, 2007)

## 1.6 Nutritional Influences on DNA Methylation

In addition to the overexpression of DNMTs, it has also been shown that nutritional influences in the gestational period can affect DNA hypermethylation. Investigations in rats have demonstrated that raising gestational availability of folate or choline leads to increased levels of the methyl group donor SAM, causing DNA hypermethylation in liver and brain (Blusztajn *et al.*, 1998; van Engeland *et al.*, 2003; Napoli *et al.*, 2008; Kovacheva *et al.*, 2009). Further studies showed that prenatal choline deficiency decreases SAM levels, but surprisingly also leads to DNA hypermethylation in liver and brain by upregulation of *Dnmt1* (Kovacheva *et al.*, 2007).

SAM can be synthesized from the nutrients folate (in the form of 5-methyltetrahydrofolate) and choline. The pathways of 5-methyltetrahydrofolate and choline are metabolically interrelated at the point that homocysteine is methylated to form methionine, as shown in figure 8 (Newberne and Rogers, 1986; Zeisel and Niculescu, 2006). Methionine is then converted to S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT). SAM is the active methylating agent for many enzymatic methylations, including the methylation of cytosines and the sequential methylation of phosphatidylethanolamine to form phosphatidylcholine (Ridgway and Vance, 1988).



**Figure 8. Choline, folate, and methionine metabolism are interrelated**

CHD: choline dehydrogenase; BHMT: betaine homocysteine methyltransferase; MAT: methionine adenosyltransferase; SAM: s-adenosyl-L-methionine; PEMT: phosphatidylethanolamine methyltransferase; SAH: s-adenosyl-homocysteine; MTHF: 5-methyltetrahydrofolate; MTHFHM: 5-methyltetrahydrofolate-homocysteine methyltransferase; B 12, vitamin B 12; THF: tetrahydrofolate

## 1.7 Conditional Transgenes for Overexpression Based on the Cre-*loxP* Mechanism

An elegant way to investigate the *in vivo* effect of an overexpression of a gene is to study transgenic animals. The analysis of overexpression becomes problematic when the resulting changes are lethal, especially when the transgenic animals die at an early stage of development. In this case, the use of a conditional transgene is a solution to circumvent the lethal effects of the transgene.

Cre-*loxP* conditional transgene expression can be activated by crossing in a mouse strain that expresses the Cre recombinase. Various transgenic mouse strains which express constitutively or conditionally active Cre recombinase are available but the promoters or mechanisms that control the Cre recombinase expression differ. Because of this, the Cre recombinase can be expressed in specific tissues, at specific time points during development, or activated upon triggering by supplying the transgenic animals with an exogenous agent by injection, inhalation or nutritional supplements. By choosing the appropriate Cre recombinase transgenic mouse line for cross-ins with the mouse line carrying the gene of interest, the gene of interest is activated according to the expression of the Cre recombinase.

The Cre-*loxP* mechanism originates from the coliphage P1 and encodes an efficient site-specific recombination system consisting of a 38 kDa protein called cyclization recombination (Cre) and a short asymmetric DNA sequence called *loxP* (locus of X-over P1) (Sternberg and Hamilton, 1981; Abremski *et al.*, 1983; Hoess and Abremski, 1985). The *loxP* site consists of an 8 bp sequence flanked by two sets of palindromic 13 bp sequences (Fig. 9).

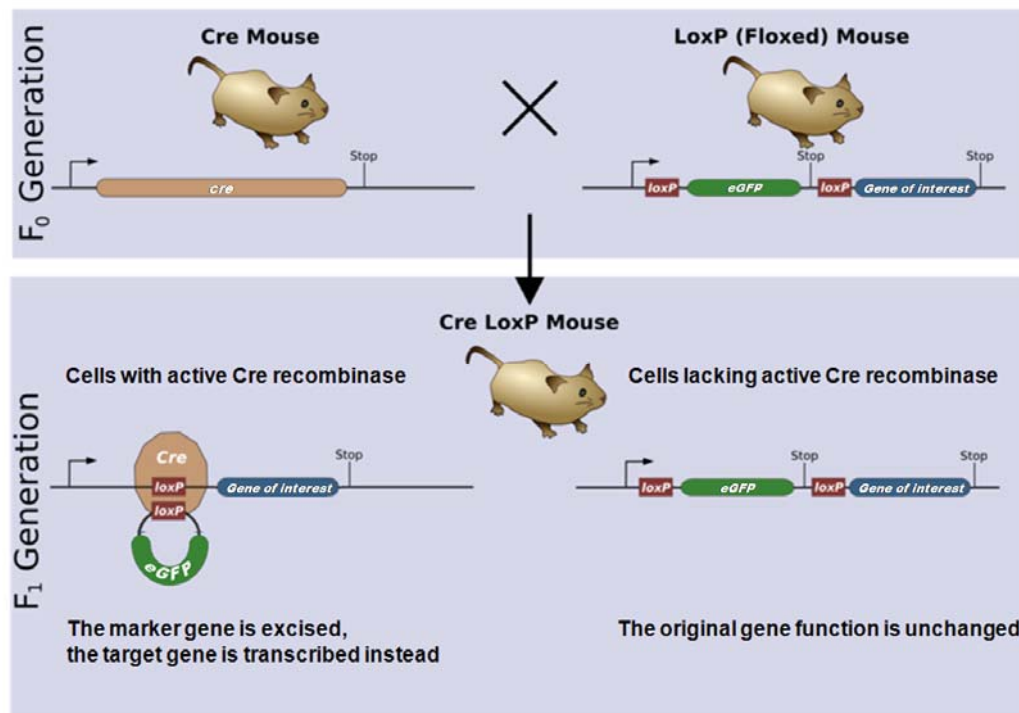


**Figure 9. Detailed structure of the *loxP* site**

The *loxP* site consists of an 8 bp sequence flanked by two palindromic 13 bp sequences.

Recombination between two *loxP* sites of the same orientation occurs when the sites are present on either supercoiled or linear DNA, and is independent of the relative orientation of the *loxP* sites on the DNA (Sauer, 1987). The recombination between two directly repeated sites on the same chromosome results in a deletion of the DNA segment lying between the sites, whereas the recombination between two sites of inverted orientation results in an inversion of the DNA segment between the two sites (Sauer and Henderson, 1988). The only factor necessary to trigger the recombination of *loxP* sites is the Cre recombinase protein. The Cre protein consists of 343 amino acids and is composed of four subunits and two domains. The catalytic site of the enzyme is the C-terminal domain, which is similar in structure to the domain in the Integrase family of enzymes isolated from lambda phage (Sauer, 1987).

By flanking or “floxing” a gene region with *loxP* sites it is possible to target this region for site-specific recombination by the Cre recombinase. This mechanism can be used to create a conditional transgene for overexpression that is only activated when the cells carrying the transgene are exposed to the Cre recombinase. Two separate transgenic mouse strains are needed to create an *in vivo* mouse model for the conditional expression of a transgene, one carrying the Cre recombinase, and the other carrying the transgene with the gene of interest. A floxed transcriptional pause site must be inserted between the promoter and the gene of interest for the latter transgene so that the expression of the gene of interest is not initially driven by the promoter. As in this study, a marker protein cassette can be added to the transcriptional pause site to facilitate the detection of the non-recombined transgene. Upon exposure to Cre recombinase the marker protein and transcriptional pause site sequences are excised and the promoter and the gene of interest are brought together, resulting in the expression of the gene of interest (Fig. 10).



**Figure 10. A model experiment using the Cre-loxP Mechanism**

In the F<sub>0</sub> Generation there are two separate mouse strains, one carrying a gene for the Cre recombinase (top left), and the other carrying a transgene consisting of a gene of interest which is preceded by a floxed marker protein (eGFP) (top right). Cross-ins from the two strains that carry the transgene and the Cre recombinase gene (bottom left) are subject to recombination in cells where both genes are present, resulting in the excision of the marker protein and the expression of the gene of interest. Cross-ins that do not carry the Cre recombinase gene continue to express the marker gene, as in the F<sub>0</sub> generation (bottom right). (Figure adapted from Matthias Zepper, 2008; [http://commons.wikimedia.org/wiki/File:CreLoxP\\_experiment.png](http://commons.wikimedia.org/wiki/File:CreLoxP_experiment.png), licensed under the Creative Commons Attribution ShareAlike 3.0)

## 1.8 Aim

The aim of this project is to create a mouse model which conditionally overexpresses methyltransferases. Transgene constructs for the somatic isoform of the maintenance DNA methyltransferase *Dnmt1*, *Dnmt1s*, as well as the histone methyltransferase G9A, are to be prepared. The transgene construct should furthermore allow the exchange of these methyltransferases for other DNA- or histone-methyltransferases.

Starting with tests on the *Dnmt1* transgene before the establishment of a G9A transgenic line, this project focuses on the creation of a mouse model to study the consequences of *Dnmt1* overexpression. Because previous studies have found that overexpression of *Dnmt1* was embryonically lethal, a conditional approach using the Cre-loxP mechanism is adopted. The vast spectrum of Cre-recombinant mouse lines is expected to verify previous *Dnmt1* overexpression studies using a ubiquitous expression and provide a basis for an analysis of the consequences of *Dnmt1* overexpression in specific tissues or during specific periods of development. Using this strategy, the gain-of-function *Dnmt1s* mouse model should provide a powerful tool to determine whether an overexpression of *Dnmt1s* alone is sufficient to trigger hypermethylation of susceptible parts of the genome and, if so, what the consequences of these epimutations are. Following previous publications, the effect of ubiquitous *Dnmt1s* overexpression on the expression of *Igf2* is investigated in this study. Because a viable transgenic mouse model for the *in vivo* overexpression of *Dnmt1* has not been reported so far, the *Dnmt1s* mouse model may well be a valuable system for the investigation of the effects of *Dnmt1s* overexpression in many fields of research.



## 2. Materials and Methods

The following procedures were taken from the methods collection of Sambrook and Maniatis (1989), unless other sources are given as reference.

### 2.1 Materials

#### 2.1.1 Chemicals, Enzymes, and Solutions

The non-radioactive chemicals and enzymes were purchased from Biomers (Ulm) Boehringer Mannheim (Mannheim), Clontech (Heidelberg), Invitrogen (Karlsruhe), Merck (Darmstadt), New England Biolabs (NEB), Promega (Mannheim), Roth (Karlsruhe), Sigma (Heidelberg), Serva (Heidelberg), GibcoBRL (Eggenstein), MBI Fermentas (St. Leon-Rot), Peqlab (Erlangen), BD (Heidelberg), Roche (Mannheim) and MWG (Ebersberg) in *pro analysis* quality, unless stated otherwise.

Radioactive chemicals were purchased from Perkin-Elmer (Rodgau – Jügesheim).

#### 2.1.2 DNA and Protein Markers

DNA Markers:        1 kb DNA ladder, Invitrogen  
                          100 bp DNA ladder, Invitrogen  
                          pUC19 *MspI*-digested, Fermentas  
                          FastRuler™ DNA ladder, Low Range, Fermentas

Protein Marker:     PageRuler™ Plus Prestained Protein Ladder, Fermentas

#### 2.1.3 Oligonucleotides

All oligonucleotides or primers were created with the Primer3 software ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)). The individual sequences are listed in the Appendix.

### 2.1.4 Plasmids

pVL1393- <i>Dnmt1s</i>	pVL1393 vector containing the <i>Dnmt1s</i> sequence
pLCMV- <i>ECFP-loxP-FRT</i>	Vector containing the <i>eCFP</i> sequence
pEGFP-N3- $\Delta$ Not	Vector containing the <i>eGFP</i> sequence
pCX-FLAG-P/CAF	Vector containing the CAG promoter
pCL-Cre	Vector for Cre-recombinase expression

### 2.1.5 Bacterial Strains

<i>E. coli</i> K12 GM2163	$F^-$ , <i>ara</i> -14, <i>leu</i> B6, <i>fhu</i> A31, <i>lac</i> Y1, <i>tsx</i> 78, <i>gln</i> V44, <i>gal</i> K2, <i>gal</i> T22, <i>mcr</i> A, <i>dcm</i> -6, <i>his</i> G4, <i>rfb</i> D1, <i>rps</i> L136, <i>dam</i> 13::Tn9, <i>xyl</i> A5, <i>mtl</i> -1, <i>thi</i> -1, <i>mcr</i> B1, <i>hsd</i> R2
<i>E. coli</i> DH5 $\alpha$	$F^-$ , <i>end</i> A1, <i>hsd</i> R17, ( $r_k^- m_k^+$ ), <i>sup</i> E44, <i>thi</i> -1, <i>rec</i> A1, <i>gyr</i> A, (Nal <sup>r</sup> ), <i>rel</i> A1, D( <i>lac</i> ZYA- <i>arg</i> F)U169, <i>deo</i> R, ( $\Phi$ 80d <i>lac</i> Z $\Delta$ M15)

### 2.1.6 Cell Line

NIH-3T3	murine fibroblast cell line (ATCC 1658, adherent, DMEM)
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### 2.1.7 Mouse Strains

C3H	used for oocyte donation, Model 025, Charles River
C57BL/6	surrogate mother and background for the transgenic mice strains, Model 027, Charles River
CMV-Cre	activator strain for the ubiquitous activation of the transgene

### **2.1.8 Antibodies**

Mouse anti Dnmt1 antibody [60B1220] (Abcam, Catalog-No. ab13537)

Mouse anti RGS(H)4 antibody, BSA-free (Qiagen, Catalog-No. 34650)

Stabilized Goat Anti-Mouse HRP Conjugated (PIERCE, Catalog-No. 1858413)

### **2.1.9 Enzymes**

#### Restriction endonucleases:

All restriction endonucleases were purchased from Roche (Mannheim) or New England Biolabs (Frankfurt am Main).

#### Other enzymes:

*AmpliTaq* Gold polymerase (Roche)

GoTaq Green polymerase (Promega)

T4-DNA-Ligase (Roche)

HotstarTaq (Qiagen)

## 2.2 Methods

### 2.2.1 General DNA and RNA Procedures

#### 2.2.1.1 Mini-Preparation of Plasmid DNA

For mini-preparations of plasmid DNA, a single bacterial colony (*E. coli* strain DH5 $\alpha$  or *E. coli* strain K12 GM2163) was incubated overnight in 5 ml LB with ampicillin (100  $\mu$ g/ml) at 37 °C and 250 rpm. 1.5 ml of the overnight culture were transferred to a 1.5 ml reaction tube and pelleted for 5 min (2600 x g, 4 °C). The pellet was resuspended in 300  $\mu$ l buffer P1 and mixed by vortexing. 300  $\mu$ l buffer P2 were added, the sample vigorously mixed by shaking, and then incubated at RT for 3 min. 300  $\mu$ l buffer P3 were added, the sample vigorously mixed by shaking, centrifuged for 15 min (10300 x g, 4 °C), and placed on ice immediately after centrifugation. The supernatant was transferred to a fresh 1.5 ml reaction tube containing 500  $\mu$ l ethanol (100 %) and centrifuged for 20 min (16000 x g, RT). The DNA pellet was washed with 70 % ethanol and centrifuged for 5 min (16000 x g, RT). The supernatant was discarded and the pellet left to air-dry for 15 min before resuspension in 25  $\mu$ l H<sub>2</sub>O.

Buffer P1	10 mM	EDTA
	50 mM	Tris-HCl, pH 8,0
	100 $\mu$ g/ml	RNase A
Buffer P2	0.2 M	NaOH
	1 %	SDS
Buffer P3	3 M	potassium acetate
	adjusted to pH 5.5 with HAc	

### 2.2.1.2 Maxi-Preparation of Plasmid DNA

Maxi-preparations of plasmid DNA were performed using the Plasmid Maxi Kit (Qiagen), strictly following the protocol for low-copy plasmids.

### 2.2.1.3 Agarose Gel Electrophoresis

DNA or PCR products were separated by size on 1 - 2 % (w/v) agarose gels. The agarose was boiled in TAE buffer, cooled at RT for 5 -10 min, and 0.4 µg/ml ethidium bromide (EtBr) was added. Gels were run in TAE buffer with 0.4µg/ml EtBr. 6x loading dye were added to the samples before application to the gel slots. The separation was performed at 60 V for Southern blots and at 100 – 120 V for all other applications. DNA Markers were used to determine the specific sizes of fragments. The EtBr, which intercalates into the DNA, enabled visualization of the DNA on a UV-transilluminator with a wavelength of  $\lambda=312$  nm.

6x DNA Loading Dye	1.5 g	Ficoll 400 (Amersham)
	2.5 mg	Bromphenol blue (Merck)
	2.5 mg	Xylene cyanol FF (Merck)
	200 µl	0,5M EDTA Solution
	ad 10 ml	H <sub>2</sub> O (Merck)
10x TAE Buffer	48.4 g	Tris Base (Sigma)
	11.4 ml	Acetic Acid (J.T. Baker)
	20 ml	0.5M EDTA Solution
	ad 1l	H <sub>2</sub> O (Merck)
1 kb DNA-Ladder	50 µl	1 µg/µl 1 kb Ladder (Invitrogen)
	100 µl	6x DNA Loading Dye
	850 µl	H <sub>2</sub> O (Merck)

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pUC19 DNA (MspI(HpaII) digested)	100 µl	0.5 µg/µl pUC19 (Fermentas)
	200 µl	6x DNA Loading Dye
	900 µl	H <sub>2</sub> O (Merck)

#### 2.2.1.4 Gel Extraction of DNA

After electrophoresis, DNA bands were excised from the agarose gel on a UV-transilluminator with a wavelength of  $\lambda=312$  nm. The QIAquick Gel Extraction Kit or the MinElute Gel Extraction Kit (both Qiagen) were used to purify the DNA from the gel slice. The procedure was performed strictly according to the manufacturer's protocol.

#### 2.2.1.5 Concentration Measurements of DNA and RNA in Solution

The exact concentration of DNA and RNA in solution was determined by photometric adsorption measurements at a wavelength of  $\lambda = 260$  nm and 280 nm in a ND-1000 Spectrophotometer (NanoDrop Technologies). An  $OD_{260} = 1$  corresponds to a dsDNA concentration of 50 µg/ml or an RNA concentration of 40 µg/ml. The ratio of  $OD_{260}$  to  $OD_{280}$  indicates the purity of the sample. The ratio is 1.8 for pure DNA and 2.0 for pure RNA.

#### 2.2.1.6 Restriction Digests

For analytical and preparative purposes, DNA or plasmid DNA was digested in a ratio of 1 µg DNA / 10 U restriction enzyme in the buffer supplied by the manufacturer in a total volume of 20 µl, or 50 µl for the Southern Blot procedure. Digests were performed at 37 °C for 2 – 20 h. The volume of restriction enzyme was never above 10 % of the total volume to prevent high glycerol concentrations from inhibiting the digest.

### 2.2.1.7 PCR Amplification of DNA

The Polymerase Chain Reaction procedure, first described by Mullis *et al.* in 1986, was used to amplify specific regions from plasmid DNA, genomic DNA and cDNA.

Reactions were performed in volumes of 25 or 50  $\mu$ l, with either AmpliTaq Gold polymerase (Roche, Applied Biosystems) or GoTaq Hot Start polymerase (Promega). In general, each preparation contains PCR buffer (supplied with the polymerase),  $MgCl_2$  (if  $MgCl_2$ -free buffer was used), 400-1000 nM specific 5'- und 3'-oligonucleotides (Biomers), hereinafter referred to as "primer(s)", 200-500  $\mu$ M dNTP-mix, 50 – 1000 ng DNA as template, and 0.1 – 0.2 U/ $\mu$ l polymerase. The thermocycler programs and specific sequences for each primer are listed in the Appendix.

### 2.2.1.8 DNA Sequencing Procedure

#### 2.2.1.8.1 Preparation Sequencing Reactions

The sequencing of cloned PCR-products was performed using the "Big Dye Terminator Cycle Sequencing Kit v1.1" (Applied Biosystems). A 20  $\mu$ l sequencing reaction was prepared. The sequencing reaction and program were as follows:

Table 1. Sequencing reactions

Reaction volume	20 $\mu$ l
PCR-Product (200 - 500 bp)	5-10 ng
Ready Reaction Premix*	4 $\mu$ l
5x BigDye Sequencing Buffer*	2.5 $\mu$ l
Primer (5 $\mu$ M)	2 $\mu$ l
H <sub>2</sub> O (Merck)	ad 20 $\mu$ l

\* "Big Dye Terminator Cycle Sequencing Kit v1.1"

Table 2. Thermocycler program for sequencing reactions

96 °C	1 min	Hold
96 °C	10 s	} 25 cycles
55 °C	5 s	
60 °C	4 min	

### 2.2.1.8.2 Purification of Sequencing Reactions

The products from sequencing reactions were purified with sephadex columns. Sephadex G-50 fine (GE Healthcare) was filled into the MultiScreen 45 µl Loader (Millipore), transferred to a MultiScreen<sub>HTS</sub>-HV plate, and soaked in 300 µl H<sub>2</sub>O (Merck) per well. Excess H<sub>2</sub>O was drained by centrifugation at 2.750 rpm (Centrifuge 5403, 16M 2-MT, Eppendorf), and the plate washed twice with 150 µl H<sub>2</sub>O (Merck).

The purification of sequencing reactions was then performed as follows:

Short protocol:

- Add 1 µl 2.2 % (w/v) SDS to each reaction, heat to 98 °C for 5 min
- Dilute with 20 µl H<sub>2</sub>O (Merck), transfer reactions to sephadex plate
- Centrifuge (5 min, 2750 rpm, Centrifuge 5403, 16M 2-MT, Eppendorf)
- Add 20 µl Hi-Di Formamid<sup>TM</sup> (Applied Biosystems)

### 2.2.1.8.3 DNA Sequencing

Purified sequencing reactions were analyzed with a 3100 Genetic Analyzer (Applied Biosystems). Results were analyzed with Sequencing Analysis 3.7 (Applied Biosystems) and Sequencher 4.5 (Gene Codes Corporation) software.

Dye Set: E (Big Dye® Kit v1.1)  
 Mobility File: DT3100POP6{BD}v2.mob  
 Run Module: Seq50\_20s\_6500s  
 Analysis Module: BC\_3100POP6SR\_seqOffFtOff.saz



### 2.2.2 Transgene Cloning

The cloning of the transgenes containing the N-terminally RGS-His-tagged (amino acid sequence: RGSHHHHHH) murine *Dnmt1s* and the HA-tagged (amino acid sequence: YPYDVPDYA) human *G9A* sequences were performed by Lothar Vaßen from the “*Institut für Zellbiologie*” (IFZ), the Institute for cell biology of the University Hospital, Essen. The *Dnmt1s* cDNA was kindly provided by S. Pradhan from New England Biolabs. The *G9A* cDNA was provided by M.S. Horwitz from the Department of Genome Sciences of the University of Washington School of Medicine.

#### 2.2.2.1 Cloning of pCAG-eGFP-loxP-RGS-His-*Dnmt1s*

In brief, the RGS-His-tagged *Dnmt1s* was initially cloned into the pLCMV-eCFP-loxP plasmid resulting in the pLCMV-eCFP-loxP-*Dnmt1s* plasmid. As the eCFP marker protein was not easy to detect and the transgene showed no expression (data not shown), the eCFP region of the pLCMV-eCFP-loxP-*Dnmt1s* plasmid was replaced by the eGFP sequence of the pEGFP-N3-ΔNot plasmid to facilitate the detection of the fluorescent marker protein. Furthermore, the CMV promoter was replaced by the CAG promoter from the pCX-FLAG-P/CAF plasmid in order to achieve a stronger ubiquitous expression of the transgene, resulting in the final version of the transgene plasmid pLCAG-eGFP-loxP-*Dnmt1s*.

#### 2.2.2.2 Cloning of pCAG-eGFP-loxP-HA-*G9A*

In short, the HA-tagged *G9A* was initially cloned into the pLCMV-eCFP-loxP plasmid resulting in the pLCMV-eCFP-loxP-*G9A* plasmid. To replace the CMV promoter and eCFP sequences with the CAG promoter and eGFP sequence, respectively, the *Dnmt1s* sequence was removed from the pLCAG-eGFP-loxP-*Dnmt1s* plasmid from 2.2.2.1 to create an empty pLCAG-eGFP-loxP vector. Then the *G9A* transgene sequence was cut out from the pLCMV-eCFP-loxP-*G9A* plasmid and was cloned into

the empty pLCAG-*eGFP-loxP* vector, resulting in the final version of the transgene plasmid pLCAG-*eGFP-loxP-G9A*.

### 2.2.3 Bacteria

#### 2.2.3.1 Bacterial Media

b-Medium	5 g Yeast extract 20 g Bacto-Tryptone 5 g MgSO <sub>4</sub> adjusted to pH 7.6 with KOH
LB-Medium	1 % NaCl 1 % Bacto-Tryptone 0.5 % Yeast extract adjusted to pH 7.5 with NaOH
LB-Agar	15 g/l Bacto-Agar in LB-medium
Tfbl Buffer	30 mM KOAc 100 mM RbCl 10 mM CaCl <sub>2</sub> 50 mM MnCl <sub>2</sub> 15 % Glycerine adjusted to pH 5.8 with 0.2 M HAc
TfblI Buffer	10 mM MOPS 75 mM CaCl <sub>2</sub> 10 mM RbCl <sub>2</sub> 15 % Glycerine adjusted to pH 6.5 with KOH
Ampicillin	final concentration 100 µg/ml

### 2.2.3.2 Production of Competent Bacteria

Bacterial cells that can take up DNA from the surrounding medium (competent cells) were produced using a slightly modified version of the rubidium-chloride method (Maniatis *et al.*, 1982). A single colony of the *E. coli* strain DH5 $\alpha$  or *E. coli* strain K12 GM2163 was incubated in 10 ml b-medium at 37 °C and 250 rpm until an OD<sub>550</sub> of 0.3 was reached. 5 ml of this culture were transferred into 100 ml b-medium and incubated at 37 °C and 250 rpm. At an OD<sub>550</sub> of 0.48 the culture was split into four 25 ml aliquots in pre-cooled 50 ml falcon tubes and incubated on ice for 5 min. The cells were then spun down at 1250 x g for 15 min at 4 °C, and the pellet was carefully resuspended in 10 ml ice-cold TfbI buffer. After a 90 min incubation on ice the cells were spun down again at 1250 x g for 15 min at 4 °C, and the pellet was resuspended in 1 ml ice-cold TfbII buffer. The bacterial suspension was transferred into ice-cold 1.5 ml reaction tubes in 100  $\mu$ l aliquots, snap frozen in liquid nitrogen, and stored at -80 °C until further use.

### 2.2.3.3 Transformation of Competent Bacteria

For the transformation procedure an aliquot of competent bacteria was thawed on ice. 0.1 ml of competent bacteria was mixed with 1 – 10 ng of plasmid DNA and incubated on ice for 30 min. After incubation the mixture was heat-shocked at 42 °C for 2 min in a water bath and immediately placed back on ice. After addition of 500  $\mu$ l LB-medium the samples were incubated at 37 °C for 1 h in a shaker at 300 rpm. 100  $\mu$ l of the transformation preparation were plated out on an LB-agar plate containing ampicillin. The remainder of the transformation preparation was briefly spun down, resuspended in 100  $\mu$ l supernatant, and plated out on an LB-agar plate containing ampicillin. The plates were incubated overnight at 37 °C before picking colonies for preparation of plasmid DNA as described in 2.2.1.1.

#### 2.2.3.4 Control of Plasmid DNA

Plasmid DNA amplified by bacterial transformations was tested by digesting the DNA with *EcoRI* or *NcoI* restriction endonucleases for the *Dnmt1s* or *G9A* constructs, respectively, as described in 2.2.1.6. Digested DNA was run on an agarose gel, and plasmid DNA was only purified from the gel and used for further experiments if bands of the expected fragment sizes (*Dnmt1s*: 267 bp, 4005 bp, 6995 bp; *G9A*: 499 bp, 1060 bp, 1517 bp, 5449 bp) were obtained.

#### 2.2.4 Cell Culture

All cell culture media and supplements were obtained from GibcoBRL (Eggenstein).

##### 2.2.4.1 Cell Culture Media

DMEM (Dulbecco's Modified Eagle's Medium) pH 7.0

4.5 g/l	G-Glucose
29.23 g/l	L-Glutamin
3.7 g/l	NaHCO <sub>3</sub>

FCS (Fetal Calf Serum), heat inactivated

Culture Medium	DMEM + 10 % FCS
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Trypsin/EDTA	2.0 g/l Trypsin
	0.2 g/l EDTA
	in PBS (pH 7.2) w/o Ca <sup>2+</sup> /Mg <sup>2+</sup>

#### **2.2.4.2 Cultivation and Splitting of Cells**

NIH-3T3 cells were cultivated in 75 cm<sup>2</sup> culture flasks with 12 ml culture medium at 37 °C in an incubator at 5 % CO<sub>2</sub> and a relative humidity of 90 %. All procedures were performed under a laminar flow hood. All media and reagents were autoclaved or sterile-filtered before use.

Cell splitting was performed when cells had reached 70 – 90 % confluency. The culture medium was removed, cells washed with 10 ml PBS, and detached by incubation in 1 ml Trypsin/EDTA for 5 min in the incubator. Trypsin was inactivated by addition of 9 ml culture medium. The detached cells were transferred to a 15 ml Falcon tube, spun down for 5 min at 200 x g, and resuspended in 10 ml culture medium. Cells were split 1:10 into a new cell culture flask. Successful transfection of cell cultures was checked by replacing the culture medium with PBS in cultures and observing eGFP fluorescence under the microscope, and by western blot analysis of whole cell lysates with a RGS-His-tag specific antibody.

#### **2.2.4.3 Transient Transfection of Plasmid DNA into NIH-3T3 Murine Fibroblasts with Roti-Fect**

Roti®-fect reagent for the liposome-mediated transfection of eukaryotic cells was used for transient transfection of NIH-3T3 cells with slight alterations to the supplier's protocol. 20 µg of plasmid DNA was transferred into a 1.5 ml reaction tube and filled to 400 µl with culture medium. In a separate 1.5 ml reaction tube 90 µl of Roti®-fect were mixed with 310 µl culture medium. For co-transfections, 20 µg of each plasmid DNA was used, and the volumes of Roti®-fect and culture medium were doubled. Both tubes were combined and incubated for 30 min at RT. During incubation, cells were washed twice with PBS and 8 ml culture medium was added. After incubation, the DNA-Roti®-fect mixture was added to the cultures and mixed by gently tilting the flask. Transfected cultures were incubated for 6 h at standard conditions, washed once with PBS, and incubated in culture medium (without transfection reagent) for a

further 18 h before preparation of whole cell protein extracts. The success of the transfection was tested by fluorescence microscopy.

## **2.2.5 Protein from Cell Culture**

### **2.2.5.1 Whole Cell Protein Extracts from Transfected Cell Cultures**

To obtain whole cell protein extracts, transfected cultures were washed with PBS before being collected in 1 ml PBS with a cell scraper. The cell suspension was transferred into a weighed 1.5 ml micro centrifuge tube and centrifuged in a micro centrifuge at 13000 rpm for 1 min. The supernatant was removed and the weight of the pellet determined. The pellet was then resuspended at a concentration of 200 µg/ml in whole cell extract buffer containing protease inhibitors. Samples were briefly frozen on dry ice, thawed in a 45 °C water bath for 30 sec, and centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant containing the whole cell protein extract was transferred to a new tube and stored at -80 °C until used for further experiments.

Whole Cell Extract Buffer	30 mM	Tris-HCL, pH 8
	0.42 M	NaCl
	0.5 mM	EDTA
	20 %	Glycerol
	add Protease Inhibitor Cocktail (Pierce)	

### 2.2.5.2 Concentration Measurements of Whole Cell Protein Extracts

The concentration of whole cell protein extracts from cell culture was performed using the Coomassie Plus assay reagent (Pierce). This technique was described first by Bradford *et al.* (1976) and based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. According to the manufacturer's protocol, the reagent was mixed by inverting the bottle and the required amount transferred to a 50 ml Falcon tube and left to stand to reach RT. 33  $\mu$ l of each sample were added to 1 ml of Coomassie Plus reagent in a cuvette, inverted several times, and left to stand for 5 – 10 min. The cuvettes were inverted once more before being measured in a Spectrophotometer at OD<sub>595</sub>. If the sample OD<sub>550</sub> was above 1.5, 33  $\mu$ l of a 1:10 dilution of the sample were measured. The OD<sub>550</sub> of each sample was compared to a standard curve prepared with BSA according to Table 3.

Table 3. Preparation of standard curve for protein measurements

Standard	A	B	C	D	E	F	G	H	I
c [ $\mu$ g/ $\mu$ l]	2	1.5	1	0.75	0.5	0.25	0.125	0.025	0
H <sub>2</sub> O [ $\mu$ l]	0	125	325	175	325	325	325	400	400
BSA stock solution* [ $\mu$ l]	300 (Stock)	375 (Stock)	325 (Stock)	175 (from B)	325 (from C)	325 (from E)	325 (from F)	100 (from G)	0

\*BSA stock solution: 2 mg/ml

### 2.2.5.3 Separation of Proteins by Electrophoretic Mobility (SDS-Page) (according to Laemmli, 1970)

Protein samples were separated electrophoretically on a 6 % denaturing SDS-polyacrylamide gel, prepared according to table 4 in a Mini-Protean Gel chamber (BioRad) according to Laemmli *et al.* (1970). In short, 5x Protein buffer was added to each sample in appropriate volumes and samples were boiled at 96 °C for 5 min before application to the gel slot. Separation was performed in 1x SDS running buffer

at 15 mA until the bromphenol blue band had passed the stacking gel (~1 h). Separation was then performed at 20 mA until the desired separation had been reached (~1.5 h), using the prestained protein ladder for judgement. For immunological detection, the gel containing the separated proteins was transferred to a nylon membrane.

10 % APS	10 % Ammoniumpersulfate in H <sub>2</sub> O
20 % SDS	20 % SDS in H <sub>2</sub> O
Lower Tris Buffer	1.5 M Tris-HCl, pH 8.8 0.4 % SDS
Upper Tris Buffer	0.5 M Tris-HCl, pH 6.8 0.4 % SDS
Rotiphorese Gel30	30 % Acrylamide / 0.8 % Bisacrylamide
4x Sample Buffer	62 mM Tris-HCl, pH 6.8 2 % SDS 10 % Glycerine 5 % DTT 5 % β-Mercaptoethanol 0.025 % Bromphenol blue
10x SDS Running Buffer	1.25 M Tris-Base 2 M Glycine 1 % SDS



Table 4. 6 % SDS-Polyacrylamide gel (sufficient for 2 mini-gels)

Reagent	Stacking Gel	Resolving Gel
Rotiphorese Gel30	0.65 ml	1.62 ml
Lower Tris Buffer	-	2 ml
Upper Tris Buffer	1.25 ml	-
H <sub>2</sub> O	3.1 ml	4.38 ml
20 % SDS	25 µl	40 µl
10 % APS	25 µl	40 µl
TEMED	10 µl	20 µl

#### 2.2.5.4 Protein Transfer and Detection (Western Blot Analysis)

A semi-dry blotting chamber (BioRad) was used to transfer the separated protein from the polyacrylamide gel to a nylon membrane. Before transfer, all components (extra thick Whatman paper, nylon membrane and polyacrylamide gel with separated proteins) were soaked in transfer buffer for 5 min. The components were then stacked on the anode of the chamber in the following order: extra thick Whatman paper, nylon membrane (Amersham), polyacrylamide gel, extra-thick Whatman paper. After each layer, air bubbles were removed by gently rolling over the stack with a Pasteur pipette. The blotting chamber was carefully closed with the cathode plate and the lid, and transfer was performed for 70 min at 200 mA per blot and a maximum current of 23 V.

Successful transfer was confirmed by the presence of all bands of the prestained protein marker on the nylon membrane. After transfer the nylon membrane was washed once in TBS, stained in Ponceau Red solution for 2 min, and briefly washed in TBS to visualize protein bands to confirm the successful separation and transfer of the protein samples. Gels were then destained by washing 3 x 5 min in TBS-T before being used in the western blot. For immunological detections the non-specific binding sites on the nylon membrane were blocked for 1 h in blocking solution. The membrane was then incubated over night at 4 °C in blocking solution containing a mouse monoclonal primary antibody against the RGS-His-tag (Qiagen) at a dilution of 1:1000. The membrane was washed 4 x 5 min in TBS-T and then incubated in blocking solution with a horseradish-peroxidase-conjugated goat-anti-mouse

secondary antibody at a dilution of 1:1000 for 1 h at RT. After incubation the membrane was washed 4 x 5 min in TBS-T and then incubated in ECL Substrate (Pierce) for 5 min. The membrane was then wrapped in saran wrap and exposed to an x-ray film (Fuji) for 1 to 5 min, depending on signal intensity.

10x Transfer Buffer	58.2 g Tris-Base 29.3 g Glycine 18.75 ml 20 % SDS ad 1000 ml H <sub>2</sub> O
1x Transfer Buffer	100 ml 10x Transfer Buffer 200 ml Methanol ad 1000 ml H <sub>2</sub> O
10x TBS Buffer	12.1 g/l Tris-Base 87.7 g/l NaCl ad 1000 ml H <sub>2</sub> O
1x TBS Buffer	100 ml 10x TBS Buffer ad 1000 ml H <sub>2</sub> O
1x TBS-T Buffer	1000 ml 1x TBS Buffer 500 µl Tween-20
Blocking Solution	5 % Skim Milk Powder in TBS-T
Ponceau Red Stain	0.1 % Ponceau Red dissolved in 5 % acetic acid
ECL Kit	SuperSignal West Dura Extended Duration Substrate (Pierce)

## **2.2.6 Generation of Founder Mice by Pronucleus Injections**

Before pronucleus injections, the first 200 bp each of the promoter, the GFP, and the transgene regions were sequenced, as described in 2.2.1.8, to verify that all cloning procedures had worked correctly and that the cloned fragments were in-frame. The pCAG-eGFP-loxP-Dnmt1s plasmid was digested with *NotI*, the digested plasmid DNA separated by agarose gel electrophoresis without EtBr in gel or buffer. The 8493 bp fragment containing the transgene construct was excised from the agarose gel and purified using the Qiaquick Gel Extraction Kit (Qiagen), strictly following the manufacturer's protocol, except that the DNA was eluted in specialized pronucleus injection buffer provided by the "*Zentrales Tierlaboratorium*" (ZTL), the Central Animal Facility of the University Hospital, Essen. The pronucleus injections were then performed by Ralph Waldschütz and Wojciech Wegrzyn of the ZTL: The purified transgene constructs were injected into oocytes of C3H mice, and the oocytes implanted into C57BL/6 surrogate mother animals. The resulting transgenic offspring were used as founder mice and crossed back into a C57BL/6 background.

## **2.2.7 Analysis of Transgenic Mouse Lines**

### **2.2.7.1 Microscopic Analysis of GFP Expression in Mouse Tail Biopsies**

Using a Zeiss Axioplan microscope (Zeiss), fresh tail biopsies from each mouse were observed for GFP fluorescence before being used for DNA preparations.

### **2.2.7.2 DNA Preparation from Mouse Tail Biopsies**

DNA was prepared from fresh mouse tail biopsies using the EZ1 robot system (Qiagen) with the EZ DNA Tissue Kit (Qiagen). 190 µl buffer G2 and 10 µl Proteinase K were added to the mouse tails, and samples were digested over night at 56 °C and 800 rpm in a thermoshaker (Eppendorf). The samples were then spun down at 300 x g in a microcentrifuge and the supernatant was transferred to a 2 ml

reaction tube. Finally, the DNA was isolated, strictly following the EZ DNA Tissue Kit manual.

### 2.2.7.3 Transgene Sequencing

The transgenic sequence from a non-recombined transgenic mouse was amplified in two parts using PCR (primers in appendix). The amplified fragments were run on an agarose gel, cut out, purified using the Qiagen MinElute Gel Purification Kit and prepared for sequencing as described in 2.2.1.8.1 – 2.2.1.8.2. The transgene was then sequenced as described in 2.2.1.8.3 in at least two-fold coverage using a battery of primers (sequencing primers in appendix).

### 2.2.7.4 Southern Blot for Analysis of Transgene Insertion

#### 2.2.7.4.1 Generation of the Southern Blot Probe

The DNA probe for the Southern blot procedure was generated by PCR using plasmid DNA from a maxi-preparation of the pLCAG-eGFP-loxP-*Dnmt1s* plasmid as template (Tab. 5 and 6). Twelve 50 µl PCR reactions were performed to yield enough product for several hundred Southern blots. The PCR products were run on an agarose gel, excised, and purified as described in 2.2.1.3 – 2.2.1.4. The purified PCR product was used as hybridization probe in Southern blots for mice carrying the *Dnmt1s* transgene.

Table 5. Thermocycler program for Southern blot DNA probe

95 °C	3 min	hold
95 °C	25 s	} 35 cycles
56 °C	25 s	
72 °C	2 min	
72 °C	10 min	hold
4 °C	∞	hold

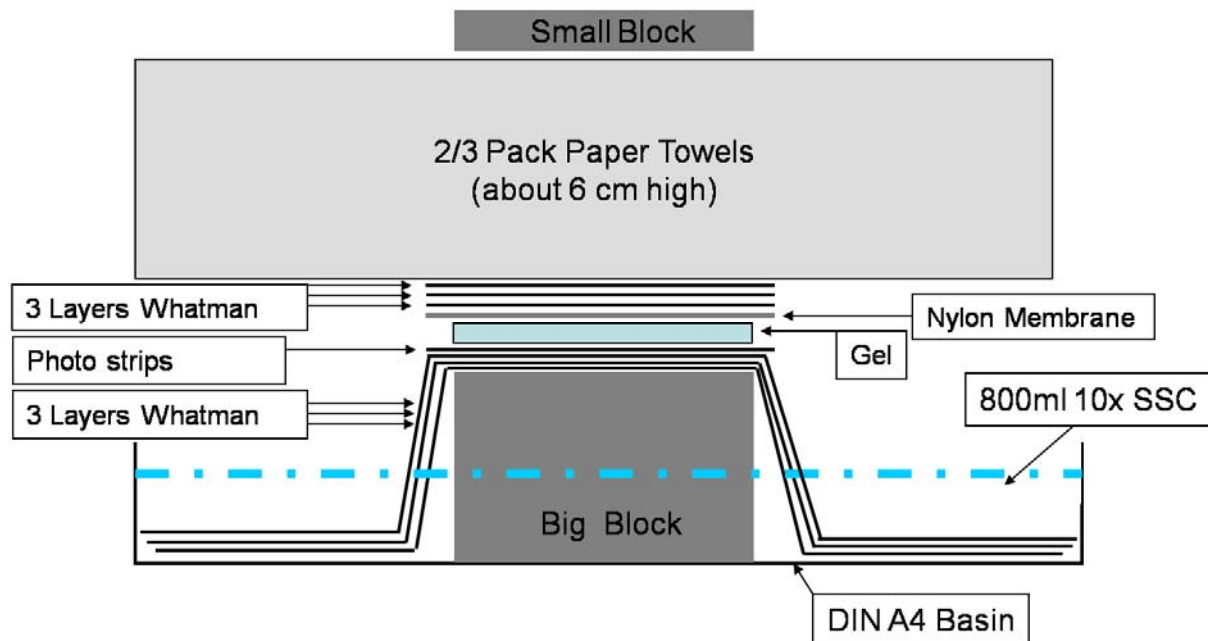
Table 6. PCR reaction to generate Southern blot probe

	1 reaction [ $\mu$ l]	12 reactions [ $\mu$ l]
H <sub>2</sub> O	33.5	402
10x Buffer	5	60
MgCl <sub>2</sub>	3	36
DMSO	2.5	30
dNTP mix [10 $\mu$ M each]	2	24
Dnmt1_g_US3 [100 $\mu$ M] (Forward Primer)	0.5	6
Dnmt1_g_LS3 [100 $\mu$ M] (Reverse Primer)	0.5	6
AmpliTaq Gold Polymerase	1	12
DNA Maxiprep	2	24
Total	50	

#### 2.2.7.4.2 Southern Blot procedure (according to Southern, 1975)

4  $\mu$ g DNA from mouse tail biopsies were digested overnight with *Eco*RI as described in 2.5.6. The complete digest was run on a 0.8 % agarose gel at 60 V for 5 - 6 h using a 1 kb ladder in the far left lane to check the running distance. After the run, a picture of the gel with a fluorescent ruler was made on a UV-transilluminator with a wavelength of  $\lambda=312$  nm before cutting off the far left lane containing the 1 kb ladder. The gel was then denatured by gentle shaking for 30 min in denaturing solution. After denaturing the gel was stacked into a Southern blot according to Fig. 11 and blotted over night. In short, a big block, the size of an agarose gel and 4 cm high, was placed in a DIN A4 basin containing 800 ml 10x SSC. Three layers of Whatman paper (35 cm x 15 cm), pre-wet in 4xSSC, were layered over the block. Air bubbles were removed with a glass rod after each new layer. The denatured gel was placed on top of the three layers of Whatman paper, and a frame of strips cut from developed x-ray film was laid around the gel. A nylon 66 blotting membrane (type B, positive, Fluka BioChemika) pre-wet in 4x SSC was placed on the gel and air bubbles were removed with a glass rod. Three more layers of Whatman paper (10 x 10 cm), pre-wet in 4x SSC, were layered over the block, removing any air bubbles with a glass rod after each layer. A 6 cm stack of paper towels was placed on top of the blot stack and weighted down with a 1 cm high block, which was the size of an agarose gel. The

Southern blot was left untouched for at least 16 h to ensure a complete transfer of the DNA from the gel to the membrane.



**Figure 11. Schematic diagram of Southern blot procedure**

The diagram illustrates the stacking procedure for the overnight blot.

After the transfer, the small block, the paper towels and the three top layers of Whatman paper were removed, taking care not to move the gel and the membrane. The gel slots were marked on the membrane using a pencil. The membrane was then removed, washed briefly in 4x SSC and laid on a new piece of Whatman paper to dry with the side that had touched the gel face up. The membrane was then crosslinked in an autocrosslinker with  $1200 \times 100 \mu\text{J}/\text{cm}^2$  energy units. For prehybridization, a 50 ml Falcon tube was prepared by piercing the lid of the tube with a large needle and filling it with 5 ml of Rapid-Hyb buffer (Amersham). The blot was cut to fit the 50 ml Falcon tube, taking care not to cut off any transferred DNA, and rolled into the tube making sure the side with the bound DNA faced inwards. The membrane was prehybridized at  $65^\circ\text{C}$  in a rotary oven in the radionuclide laboratory before adding the radioactively labelled probe.

#### **2.2.7.4.3 Radioactive Labelling of the Southern Blot Probe**

The DNA probe was radioactively labelled using the Amersham Megaprime™ DNA labelling system. In brief, 50 ng DNA-Probe, prepared as described in 2.2.7.3.1, were pipetted into a reaction tube with a screw-on cap filled to 30 µl with H<sub>2</sub>O. 5 µl primer solution were added and briefly mixed by pipetting before denaturation at 95 °C for 10 min. After denaturation, the sample was placed on ice immediately and let cool for 2 min to prevent renaturation. The sample was spun down for 1 min at 11300 x g in a microcentrifuge and placed back on ice. 10 µl labelling buffer were added and the sample mixed by pipetting. From now on all steps were carried out in the radionuclide lab. 2 µl Klenow enzyme and 5 µl α-<sup>32</sup>P-dCTP were added to the sample. The tube was placed in a pre-warmed lead container and incubated for 30 min at 37 °C. The probe was then purified using the Nucleotide Removal Kit (Qiagen). 500 µl buffer PN were added to the sample, mixed, and the solution transferred to a QiaQuick column in a 2 ml collection tube. The probe was bound to the column matrix by centrifugation at 2400 x g for 1 min. The 2 ml collection tube and its contents were discarded and the column placed in a new 2 ml collection tube. The column was washed twice with 500 µl buffer PE, centrifuging at 2400 x g for 1 min each time and discarding the 2 ml collection tube with contents after each wash. The column was placed in a new 2 ml collection tube and centrifuged dry at 11300 x g for 1 min. The column was then transferred to a fresh 1.5 ml reaction tube, and the labelled probe eluted in 100 µl buffer EB at 11300 x g for 1 min. 5 µl of the labelled probe were transferred to a 1.5 ml reaction tube with screw-on cap and measured in a scintillation counter. The rest of the probe was transferred to a reaction tube with screw-on cap and denatured for 5 min at 95 °C.

#### **2.2.7.4.4 Southern Blot Hybridization**

A volume corresponding to 5\*10<sup>6</sup> counts of radioactively labelled Southern blot probe was added to each prehybridized blot, and incubated over night at 68 °C in a rotary oven. On the next day, 2x SSC was prepared and pre-warmed to 65 °C in a water bath. The membrane was removed from the falcon tube, placed in a plastic box with

lid, covered with pre-warmed 2x SSC, and incubated in a water bath at 65 °C. After 30 min the wash solution was removed and the membrane was placed on Whatman paper and measured with a Geiger counter (Berthold LB1210B) at 10 cm distance. If a value of over 70 Bq/cm<sup>2</sup> was counted, the wash procedure was repeated. As soon as a measurement lower than 70 Bq/cm<sup>2</sup> was made, the blots were dried on Whatman paper, wrapped in saran wrap, and exposed to an x-ray film in a developing cartridge for three days at -80 °C before developing the film.

Denaturing Solution	0.25 M NaOH 0.6 M NaCl
10x SSC	876.5 g NaCl 441 g Sodium citrate 4000 ml H <sub>2</sub> O adjusted to pH 7.0 with HCL, filled to 5 l with H <sub>2</sub> O
Nylon Membrane	Fluka BioChemika Blotting-Nylon 66 Membrane, type B, positive
Hybridization Buffer	Amersham Rapid-Hyb Buffer
Radioactive Labelling Kit	Amersham Megaprime <sup>TM</sup> DNA labelling system

#### **2.2.7.5 PCR Analysis of Transgene Insertion**

Insertion of the non-recombined transgene was verified using a PCR that amplifies a 532 bp region specific for the transgene, spanning from 5' end of the CAG promoter to the 3' end of the eGFP marker protein sequence. The pipetting scheme and thermal cycler conditions are noted in the Appendix.



### **2.2.8 Crossing-in of Founder Lines with Cre-Recombinase Expressing Mouse Strains**

The six founder mice were crossed in with the CMV-Cre mouse line. The CMV-Cre mouse line expresses the Cre-recombinase ubiquitously and should thus trigger transgene recombination in all tissues.

### **2.2.9 PCR Analysis of Transgene Recombination**

Transgene recombination was analyzed using a PCR that amplifies 1299 bp and 218 bp regions specific for the non-recombined and recombined transgenes, respectively. The amplified region spans from the 5' end of the CAG promoter to the 3' end of the *Dnmt1s* transgene, resulting in a large product from non-recombined version of the transgene and a small product from recombined versions of the transgene. The pipetting scheme and thermal cycler conditions are noted in the Appendix.

### **2.2.10 Organ Extraction from Mice for RNA and Protein Analyses**

Mice designated for organ extraction were euthanized using CO<sub>2</sub>. Immediately after extraction a small piece of each organ of 50 – 100 mg was transferred to RNA*later* stabilization reagent (Qiagen) for RNA preparations. The rest of the tissue was snap frozen in liquid nitrogen for preparation of DNA or protein.

### **2.2.11 RNA Expression Analyses**

#### **2.2.11.1 RNA Preparation from Mouse Tissues**

RNA was prepared from RNA*later* (Qiagen) stabilized kidney, liver and spleen tissue using the RNeasy mini Kit (Qiagen). 10 mg of stabilized tissue were placed in 600 µl RLT buffer and disrupted and homogenized using a sonicator (Heinemann)

with a 3 mm microtip. Each sample was exposed to a 5 sec burst at 15 % power and 50 % duty cycle to prevent RNA shearing due to excessive sonication.

#### 2.2.11.2 DNase I Digest of RNA Samples

To prevent false results due to DNA contamination, the RNA samples were digested with DNase using the RQ1 RNase-free DNase Kit (Promega), strictly following the manufacturer's protocol.

#### 2.2.11.3 Reverse Transcriptase Reaction for Preparation of cDNA

RNA samples were transcribed into cDNA using reverse transcription for RT-PCR verification of transgenic RNA expression and for quantitative expression analyses of *Dnmt1* and *Igf2* using TaqMan Gene Expression Assays (Applied Biosystems). Reactions were prepared according to table 7. The reverse transcription was performed in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems) in three steps according to table 8.

Table 7. Reverse transcription

	sample	- RT control	- RNA control
RNA	1000 ng	1000 ng	-
MgCl <sub>2</sub> Solution (25 mM)*	4 µl	4 µl	4 µl
10x PCR Buffer II*	2 µl	2 µl	2 µl
dGTP (10 mM)*	2 µl	2 µl	2 µl
dATP (10 mM)*	2 µl	2 µl	2 µl
dTTP (10 mM)*	2 µl	2 µl	2 µl
dCTP (10 mM)*	2 µl	2 µl	2 µl
RNase Inhibitor (5U/µl)*	1 µl	1 µl	1 µl
Random Hexamers (50µM)*	1 µl	1 µl	1 µl
Reverse Transcriptase (50U/µl)*	1 µl	-	1 µl
H <sub>2</sub> O (Merck)	ad 20 µl	ad 20 µl	ad 20 µl

\*(Applied Biosystems)

Table 8. Thermocycler program for cDNA preparation

21 °C	10 min	Random Hexamer Binding
42 °C	15 min	Reverse Transcription
95 °C	5 min	Denaturation
4 °C	∞	hold

#### 2.2.11.4 RT-PCR for Verification of Transgenic RNA Expression

To verify the expression of RNA from the *Dnmt1s* transgene, a 209 bp fragment spanning from the RGS-His-tag into the 3' part of the transgene was amplified from cDNA using PCR.

#### 2.2.11.5 Quantitative Expression Analysis of *Dnmt1* and *Igf2* (TaqMan)

To determine the total amount of *Dnmt1* transcript (endogenous + transgenic) and the amount of *Igf2* transcript in comparison to the amount of *Gapdh* transcript, TaqMan Gene Expression Assays (Applied Biosystems) were performed (Assay IDs listed in the appendix). These Assays are a special kind of PCR, in which a probe with two fluorophores (a reporter-fluorophore and a quencher-fluorophore) binds to the target sequence between the PCR primer binding sites. As long as both fluorophores are bound, the emission of the reporter-fluorophore is quenched by the quencher-fluorophore. During the elongation phase of the PCR the probe is cleaved from the target sequence and then degraded by the 5' – 3' exonuclease activity of the Taq DNA-polymerase. This separates the reporter fluorophore from the quencher, so that its emission can be detected by a laser. By comparing the emission of the target gene to the emission of a reference gene, the relative expression of the target gene can be calculated. *Gapdh* served as reference gene for RNA from all tissues. For TaqMan analyses, 1 µg of RNA was transcribed into cDNA as described in 2.2.11.3. The reactions were pipetted together as in Tab. 9 in a 384-well plate using an EPmotion pipetting robot (Qiagen). Plates were run using the program in Tab. 10 in an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The cycle

threshold (Ct) values of each well were exported to a text file and imported to Microsoft Excel for further analysis using the Delta-Delta Ct method.

**Table 9. TaqMan Preparations**

cDNA	300 ng
TaqMan Gene Expression Assay (20x)*	1 µl
TaqMan Gene Expression Master Mix (2x)*	10 µl
H <sub>2</sub> O	ad 20 µl

\* (Applied Biosystems)

**Table 10. TaqMan Program**

50 °C	2 min	hold
95 °C	10 min	hold
95 °C	15 s	} 45 cycles
60 °C	1 min	

### 2.2.12 Whole Cell Protein Extracts from Mouse Organs

To obtain whole cell protein extracts from mouse organs, fresh or frozen (-80 °C) tissue was cut into small pieces with a scalpel and transferred to a reaction tube with whole cell extract buffer containing protease inhibitors at a concentration of 200 µg/ml. For complete lysis, the solution was disrupted and homogenized using a sonicator (Heinemann) with a 3 mm microtip. Each sample was exposed to a 10 sec burst at 15 % power and 50 % duty cycle. Samples were then centrifuged at 16000 x g for 30 min at 4 °C to pellet any remaining debris. The supernatant containing the whole cell protein extract was transferred to a new tube and stored at -80 °C until used for further experiments.

### 3. Results

Previous *in vivo* studies have shown that overexpression of *Dnmt1* is embryonically lethal (Biniszkiewicz *et al.* 2002). In order to investigate the consequences of an overexpression of *Dnmt1s* at specific time points during development or in specific tissues only, conditional transgenic mouse models were created using the Cre-*loxP* system. The expression of the transgene was activated conditionally by crossing into mouse lines that express the Cre recombinase at specific time points during development or in specific tissues only. This made it possible to breed mice that are not subjected to the early effects of *Dnmt1s* overexpression and thus create an *in vivo* model to study the consequences of *Dnmt1s* overexpression.

#### 3.1 Transgene Cloning of pLCAG-eGFP-*loxP*-Methyltransferase

To create a transgenic mouse model, the planned transgene had to be cloned and tested. The pVL1393 plasmid containing the murine *Dnmt1s* cDNA was sequenced to make sure that the primary material was intact and to exclude point mutations. Sequencing showed that the provided *Dnmt1s* sequence was correct and could be used for further cloning. The cloning of the two transgenes containing the murine *Dnmt1s* and human *G9A* sequences, respectively, were performed by Lothar Vaßen from the “*Institut für Zellbiologie*” (IFZ), the Institute for cell biology of the University Hospital, Essen. To easily distinguish between endogenous and transgenic methyltransferases, recognition tags were added to the N-terminal ends of each methyltransferase cDNA sequence. A RGS-His-tag (amino acid sequence: RGSHHHHHH) was added to the *Dnmt1s* sequence, because it has been shown that an N-terminal His-tag has no effect on Dnmt1 methyltransferase activity (Fatemi *et al.*, 2001; Hermann *et al.*, 2004; Goyal *et al.*, 2006), and a hemagglutinin-tag (HA-tag; amino acid sequence: YPYDVPDYA) was added to the *G9A* sequence. The RGS-His-tagged murine *Dnmt1s* and HA-tagged human *G9A* cDNA sequences were initially cloned into the pLCMV-eCFP-*loxP* plasmid resulting in pLCMV-eCFP-*loxP*-*Dnmt1s* and pLCMV-eCFP-*loxP*-*G9A* (Fig. 12). These transgenes were successfully tested *in vitro* using transient transfection into NIH-3T3 cells to show eCFP

expression in the original state of the transgene and expression of the tagged methyltransferase protein in the recombined version. Pronucleus injections were performed with both transgenes, but several transgenic mouse lines showed no *in vivo* mRNA or protein expression of *Dnmt1s* or *G9A* after inbreeding with Cre-recombinase expressing mouse lines (data not shown).

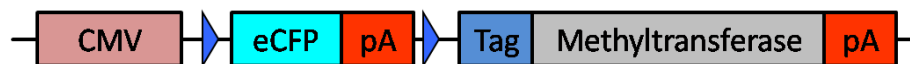
As both the *Dnmt1s* and the *G9A* transgene showed expression in the *in vitro* testing, but neither showed *in vivo* expression, it was presumed that the reason for the lack of *in vivo* expression was most likely to be due to an incompatibility of the cytomegalovirus (CMV) promoter in the *in vivo* mouse model. In consequence, new versions of both transgenes were cloned by replacing the CMV promoter of the pLCMV-eCFP-loxP-*Dnmt1s* plasmid with a CMV early enhancer/chicken  $\beta$ -actin (CAG) promoter from the pCX-FLAG-P/CAF plasmid in order to achieve a stronger ubiquitous expression of the transgene (Fig. 12). At the same time, the eCFP sequence was replaced with an eGFP sequence pEGFP-N3- $\Delta$ Not plasmid for an easier detection of the fluorescent protein from the non-recombined version of the transgene, resulting in the final version of the *Dnmt1s* transgene plasmid pLCAG-eGFP-loxP-*Dnmt1s* (Fig. 12).

To clone an equivalent version of the *G9A* transgene, the *Dnmt1s* sequence was removed from the pLCAG-eGFP-loxP-*Dnmt1s* plasmid to create an empty pLCAG-eGFP-loxP vector. The *G9A* transgene sequence was then cut out from the pLCMV-eCFP-loxP-*G9A* plasmid and cloned into the empty pLCAG-eGFP-loxP vector, resulting in the final version of the transgene plasmid pLCAG-eGFP-loxP-*G9A*. The re-cloning of the transgenes was performed by Lothar Vaßen from the “*Institut für Zellbiologie*” (IFZ), the Institute for cell biology of the University Hospital, Essen. The resulting pLCAG-eGFP-loxP-*Dnmt1s* and pLCAG-eGFP-loxP-*G9A* plasmids were sequenced to make sure that the promoter, the marker protein and the tagged methyltransferase sequences did not contain any point mutations and were in-frame. Sequencing analyses showed that both transgenes contained the CAG promoter sequence, the eGFP marker protein sequence and the tagged methyltransferase sequence without any point mutations or frame shifts, so that they could be used for further experiments.

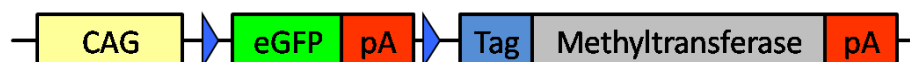
Although new versions of both the *Dnmt1s* and the *G9A* transgenes were cloned, this second attempt was initially continued with the *Dnmt1s* transgene only. This was

done to make sure that the transgene construct led to expression *in vivo* before continuing with the establishment of a G9A transgenic model.

pLCMV-eCFP-*loxP*-Methyltransferase:



pLCAG-eGFP-*loxP*-Methyltransferase:

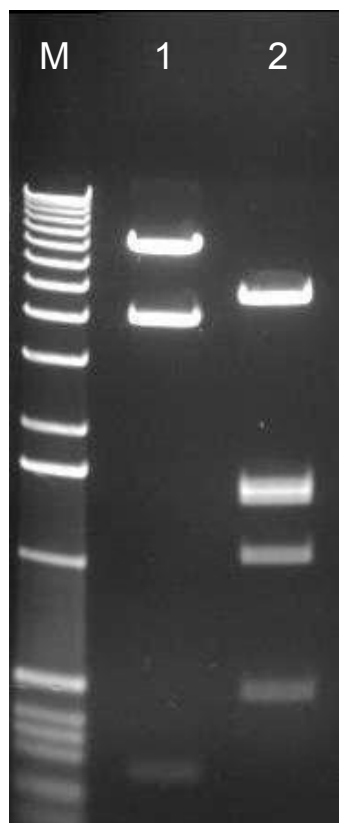


**Figure 12. Diagram of pLCAG-eGFP-*loxP*-Methyltransferase transgene re-cloning**

The initial pLCMV-eCFP-*loxP*-Methyltransferase transgene (top) was re-cloned to replace the CMV promoter and eCFP marker protein sequences with the CAG promoter and eGFP marker protein sequences, respectively, resulting in the pLCAG-eGFP-*loxP*-Methyltransferase transgene (bottom). Brown box: CMV promoter sequence; blue arrows: *loxP* sites; Cyan box: eCFP cassette; red boxes: poly A termination sequences; blue box: tag sequence; grey box: methyltransferase sequence; yellow box: CAG promoter; green box: eGFP cassette. (Figure not drawn to scale)

### 3.1.1 Restriction Digest Control of Plasmid DNA from pLCAG-eGFP-*loxP*-Methyltransferase Transformations

Plasmid DNA from pLCAG-eGFP-*loxP*-*Dnmt1s* and pLCAG-eGFP-*loxP*-G9A amplified by bacterial transformations was tested by digesting 1 µg of plasmid DNA with 20 U *Eco*RI or *Nco*I, respectively. Digested DNA was run on a 1 % agarose gel and the transgenes were only used for further experiments if bands of the expected fragment sizes (pLCAG-eGFP-*loxP*-*Dnmt1s*: 267 bp, 4005 bp, 6995 bp; pLCAG-eGFP-*loxP*-G9A: 499 bp, 1060 bp, 1517 bp, 5449 bp) were obtained. Figure 13 shows the digests of the amplified plasmid DNA containing the pLCAG-eGFP-*loxP*-*Dnmt1s* (lane 1) and pLCAG-eGFP-*loxP*-G9A (lane 2) transgenes with fragments of the expected sizes. Further testing was performed and transgenic mouse lines were established using these transgenes.



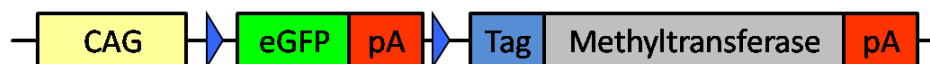
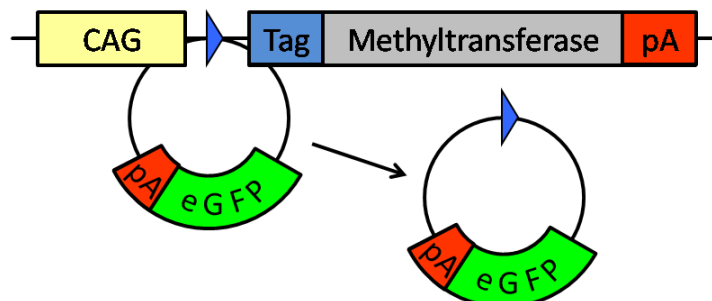
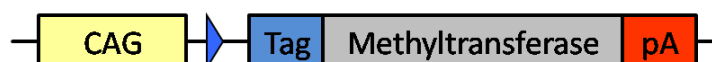
**Figure 13. Digests of *Dnmt1s* and *G9A* plasmid DNA**

Lane 1: The digest of pLCAG-eGFP-*loxP-Dnmt1s* plasmid DNA with *EcoRI* resulted in fragments of the expected sizes 267 bp, 4005 bp and 6995 bp. Lane 2: The digest of the pLCAG-eGFP-*loxP-G9A* with *NcoI* resulted in fragments of the sizes 499 bp, 1060 bp, 1517 bp and 5449 bp. The correct fragment sizes after the digest indicated an intact transgene, which was used as an indicator for further testing and use. M: Marker (1 kb ladder)

### 3.2 The Mechanism of the Transgene

The transgenes were constructed so that they initially express the eGFP marker protein. The eGFP sequences of the transgenes were floxed (Fig. 14 A). When the transgene came into contact with Cre recombinase, the *loxP* sites reacted with the Cre recombinase so that the sequence between the *loxP* sites were looped out, removed and degraded by cellular mechanisms. In the case of the transgenes in this study, this led to the excision of the eGFP marker protein and its polyadenylation signal (Fig. 14 B). Due to this deletion, the methyltransferase sequences moved close to the CAG promoter, resulting in the expression of methyltransferase in the recombined versions of the transgenes (Fig. 14 C).



**(A) Before Recombination:****(B) Recombination Event:****(C) After Recombination:****Figure 14. Transgene recombination**

(A) The CAG promoter initially drove the *eGFP* marker protein cassette resulting in *eGFP* expression. (B) Upon recombination with Cre recombinase, the floxed *eGFP* sequence was looped out and removed. It was then degraded by cellular mechanisms, preventing its re-insertion. (C) After the recombination event, the CAG promoter drove the methyltransferase cassette, resulting in expression of tagged methyltransferase. Yellow box: CAG promoter; blue arrows: *loxP* sites; green box: *eGFP* cassette; red boxes: poly A termination sequences; blue box: tag sequence; grey box: methyltransferase sequence. (Figure not drawn to scale)

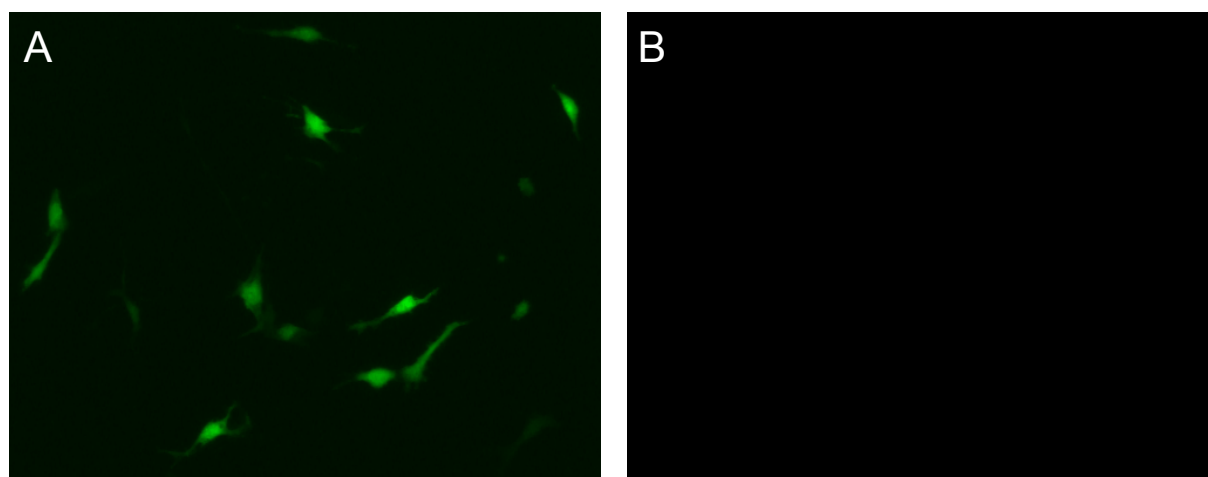
**3.3 *In vitro* Testing of Transgene Constructs**

Before the transgene was brought into a mouse strain using pronucleus injections, its functionality was tested in cell culture to avoid the integration of a non-functional transgene. This was performed by transiently transfecting pLCAG-*eGFP-loxP-Dnmt1s* plasmid DNA into the NIH-3T3 cell line to check for the expression of the *eGFP* marker protein from the non-recombined transgene by fluorescence microscopy. Co-transfection of pLCAG-*eGFP-loxP-Dnmt1s* plasmid DNA and pCL-Cre plasmid DNA was performed to verify the expression of RGS-His-tagged *Dnmt1s* protein from the recombined version of the transgene. The expression of the

recombined version of the *Dnmt1s* transgene was investigated using SDS-PAGE and western blot techniques with an antibody specific for the RGS-His-tag of the *Dnmt1s* transgene.

### 3.3.1 Transient Transfections with the pLCAG-eGFP-loxP-*Dnmt1s* Plasmid Result in eGFP Expression

The single transfections of the pLCAG-eGFP-loxP-*Dnmt1s* plasmid were controlled using a fluorescence microscope with a GFP filter. Although the transfection efficiency was relatively low (~ 50 %), transfected cells clearly showed the expression of the eGFP marker protein (Fig. 15). This proved that the promoter and eGFP fluorescent marker protein sequences were in-frame and fully functional.



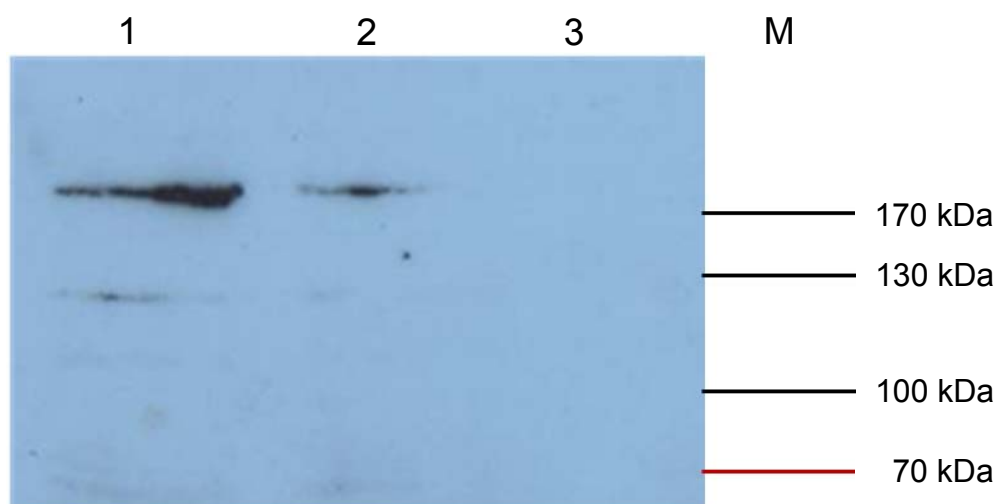
**Figure 15. eGFP expression of *Dnmt1s* transfection**

(A) Single transfection of NIH-3T3 cells with pLCAG-eGFP-loxP-*Dnmt1s* plasmid DNA showed eGFP fluorescence. (B) Co-transfection of NIH-3T3 cells with pLCAG-eGFP-loxP-*Dnmt1s* and pCL-Cre plasmid DNA showed no eGFP fluorescence.

### 3.3.2 Co-transfections of the pLCAG-eGFP-loxP-*Dnmt1s* Plasmid DNA with pCL-Cre Plasmid DNA Results in Expression of RGS-His-tagged *Dnmt1s*

The co-transfections of the pLCAG-eGFP-loxP-*Dnmt1s* plasmid DNA with pCL-Cre plasmid DNA were also controlled using a fluorescence microscope with a GFP filter. The recombination of the transgene due to the presence of the Cre recombinase led to the looping-out and degradation of the eGFP sequence. Therefore no eGFP

fluorescence could be observed in co-transfected cell cultures (Fig. 15). To show that the tagged *Dnmt1s* was being expressed in the recombined cells instead of the eGFP marker protein, an assay on the protein level was necessary. To do this, whole cell lysates were prepared from the co-transfected culture dishes, separated on an SDS-polyacrylamide gel, and probed with an RGS-His antibody using the western blot procedure. Equal loading was confirmed by a Ponceau-red stain of the blotted membrane prior to antibody incubation. The endogenous *Dnmt1s* did not contain the RGS-His-tag, so that a band detected of *Dnmt1s* size (~172 kDa) with the RGS-His antibody gave a definite indication of the expression of RGS-His-tagged *Dnmt1s* from the recombined transgene. The western blot showed a band of the expected size in co-transfections with pLCAG-eGFP-loxP-*Dnmt1s* and pCL-Cre plasmid DNA (Fig. 16, lane 1 and 2), but not in single transfections with pLCAG-eGFP-loxP-*Dnmt1s* plasmid DNA (Fig. 16, lane 3). This proved that the transgene was being successfully recombined and, as a result, RGS-His-tagged *Dnmt1s* was expressed in the co-transfected cells. It furthermore showed that the transgene did not express *Dnmt1s* until recombination with Cre recombinase had taken place, a state which would be referred to as a “leaky” conditional transgene.



**Figure 16. Western blot control of *in vitro* expression of RGS-His-tagged *Dnmt1s***

Western blot analysis with whole cell lysates of NIH-3T3 cells co-transfected with pLCAG-eGFP-loxP-*Dnmt1s* and pCL-Cre Plasmid DNA using an RGS-His-tag specific antibody. Lanes 1 and 2: transfections with different amounts (80 µg and 40 µg, respectively) of plasmid DNA. The bands of about 172 kDa in size indicated the expression of RGS-His-tagged *Dnmt1s* protein. Lane 3: The single transfection with pLCAG-eGFP-loxP-*Dnmt1s* (without pCL-Cre plasmid DNA) did not express the RGS-His-tagged *Dnmt1s* protein, showing that the transgene required recombination to express the RGS-His-tagged *Dnmt1s* and was not “leaky”. M: Marker.

### 3.4 Pronucleus Injections

The pronucleus injections were performed by Ralph Waldschütz and Wojciech Wegrzyn of the "Zentrales Tierlaboratorium" (ZTL), the Central Animal Facility of the University Hospital, Essen. A total of 26 pronucleus injections in C3H mice with the *Dnmt1s* transgene were performed. The injected oocytes were transferred to C57BL/6 females which served as surrogate mothers. The pronucleus injections resulted in six transgenic mice, of which three were female and three were male (Tab. 11). The six transgenic animals served as founder mice for the following generations and were crossed back into a C57BL/6 background.

**Table 11: Pronucleus injection success rate**

<b>CAG-eGFP-<i>Dnmt1s</i> Pronucleus Injections (f/m)</b>			
<b>Transgene</b>	<b>Transgenic</b>	<b>Wild Type</b>	<b>Total</b>
<b><i>Dnmt1s</i></b>	6 (3/3)	20 (11/9)	26 (14/12)

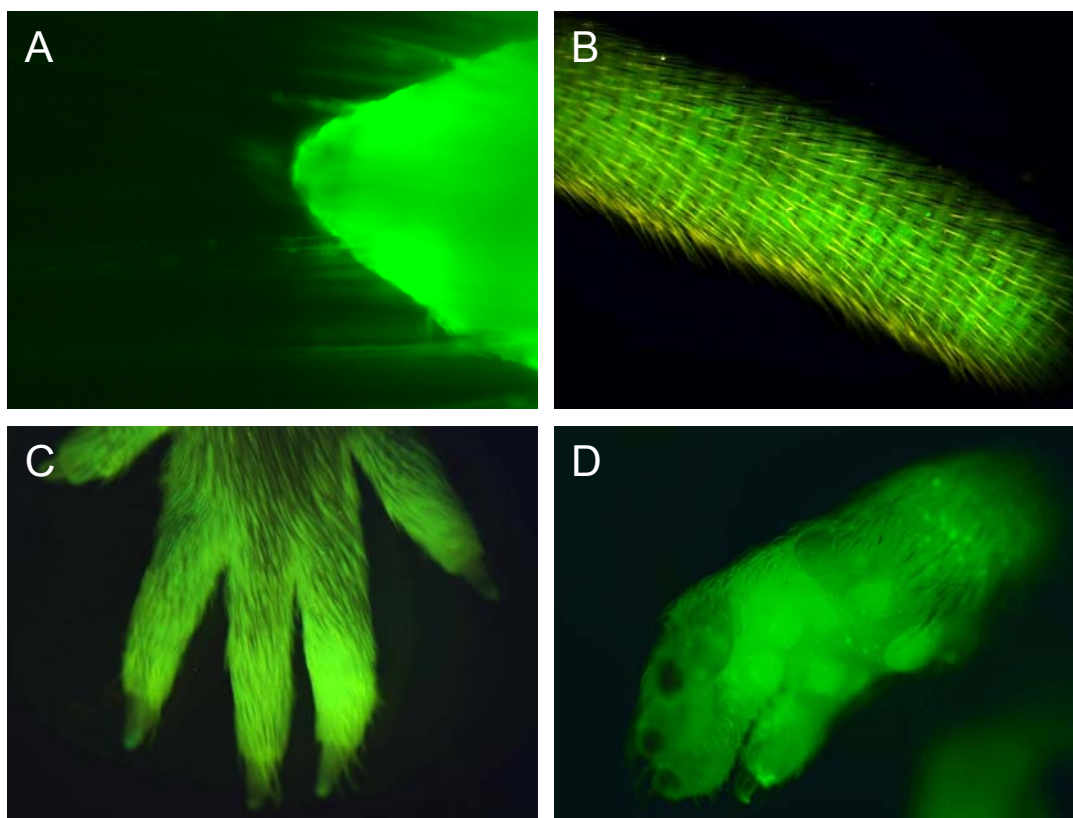
#### 3.4.1 Verification of *Dnmt1s* Transgene Sequence Integrity *in vivo* by DNA Sequencing

To make sure that the *Dnmt1s* transgene sequence had not been exposed to changes such as point mutations, the transgenic sequence from a non-recombined transgenic mouse of line 4 was fully sequenced. For this the transgenic sequence was amplified from genomic DNA in two parts, the first spanning from the CAG promoter to base 1929 the *Dnmt1s* sequence, and the second from base 1871 of the *Dnmt1s* sequence into the *Dnmt1s* polyadenylation sequence, with sizes of 3164 bp and 3299 bp, respectively. The primers for the amplification of the *Dnmt1s* cassette span over intronic sequences of the *Dnmt1s* gene to prevent amplification and sequencing of the endogenous *Dnmt1s* sequence. The PCR products were run on an agarose gel and purified using the MinElute Gel Extraction Kit (Qiagen) to remove any contaminants, such as primer dimers and genomic DNA, and prepared for sequencing as described in 2.2.1.8.1 – 2.2.1.8.2. Sequencing of the PCR amplified

fragments of the transgenic sequence revealed that there were no point mutations in any part of the transgene and that the transgenic *Dnmt1s* sequence was fully homologous to the wild type *Dnmt1s* sequence (sequencing data in appendix).

### **3.5 The eGFP Marker Protein Expressed *in vivo* from the Non-recombined Transgene**

Transgenic mice that have not been crossed in with Cre recombinant mouse strains should carry the non-recombined version of the pLCAG-eGFP-*loxP-Dnmt1s* transgene. Therefore they should ubiquitously express the eGFP marker protein. The *eGFP* expression was controlled in this study by screening the mouse tail biopsies intended for genotyping with a fluorescence microscope before the isolation of DNA. Mouse tail biopsies showed strong expression of the eGFP marker protein (Fig. 17 A and B). Fluorescence microscopy of the paws (Fig. 17 C and D), ears, nose, kidney, spleen and liver (data not shown) from the mice carrying the non-recombined transgene of lines 2 and 4 furthermore showed that the eGFP marker protein was also strongly expressed in those parts of the mouse. This proved that the eGFP marker protein was expressed in various types of mouse tissue and gave solid evidence that the CAG driven expression of the transgene is in fact ubiquitous.



**Figure 17. eGFP fluorescence of tail and paw of non-recombined transgenic mice**

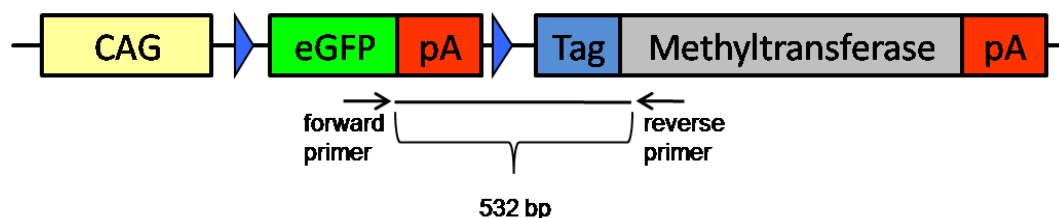
Fluorescence microscopy of mouse tails from line 2 (A) and line 4 (B) and paws from line 2 (C) and 4 (D) of transgenic mice carrying the non-recombined transgene showed the expression of the eGFP marker protein.

### 3.6 Verification of Transgene Insertion

In addition to fluorescence microscopy, molecular biological methods were performed to prove transgene insertion in transgenic mice. In this study, PCR and Southern blot were the methods of choice. The PCR provided a fast screening method to determine which mice carried the transgene. Due to its nature however, the PCR method was prone to pipetting errors or contaminations which might have resulted in false-positive results. The PCR was also sensitive to impurities in DNA preparations that could have led to false-negative results. Therefore the Southern blot method served as a control to avoid false-positive or false-negative results, as it was more sensitive due to the use of a radioactively marked probe, and less prone to errors due to the possibility of controlling each step. During pronucleus injections, the number of transgene copies that inserted into the random locus could not be

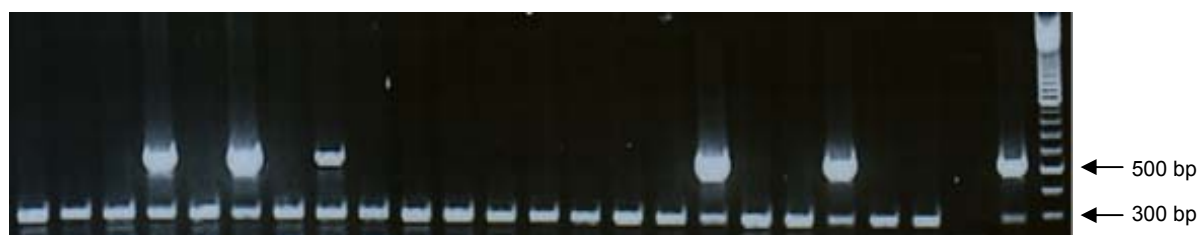
controlled. This usually resulted in more than one copy of the transgene inserting into the same locus. Due to its sensitivity, the Southern blot gave a rough indication of the number of sequential integrations of transgene copies.

The PCR to control transgene insertion used primers that spanned a 532 bp region from the *eGFP* cassette into the *Dnmt1s* sequence (Fig. 18), so that a 532 bp product indicated successful transgene insertion (Fig. 19). The PCR reaction was multiplexed with primers spanning a 295 bp region of the endogenous *Rag1* gene, so that a 295 bp product indicated a successful PCR procedure, which largely eliminated (but did not completely exclude) the possibility of a false-negative result (Fig. 19). PCR reactions lacking the 295 bp *Rag1* control fragment (Fig. 19, 3<sup>rd</sup> last lane) had to be repeated. All primer sequences are listed in the appendix.



**Figure 18. Primer positions for PCR verification of transgene insertion in founder lines**

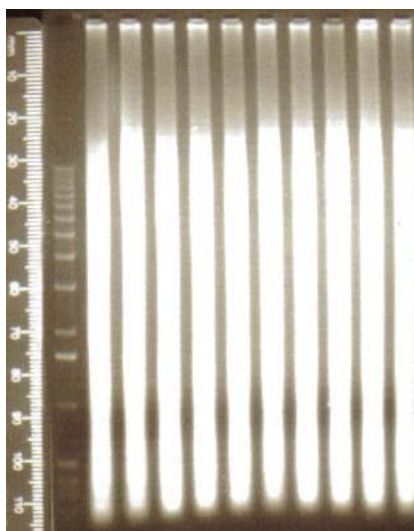
The primers spanned a 532 bp region from the *eGFP* cassette into the *Dnmt1s* sequence. (Figure not drawn to scale)



**Figure 19. PCR verification of transgene insertion**

The multiplex PCR resulted in a 295 bp control fragment from the endogenous *Rag1* gene, and a 532 bp fragment that was specific for the transgene. The 295 bp verified a successful PCR, while an additional 532 bp fragment verified the transgenic status of the mouse. Samples without the 295 bp fragment (3<sup>rd</sup> last lane) had to be repeated to exclude false-negative results. Last lane: 100 bp DNA ladder.

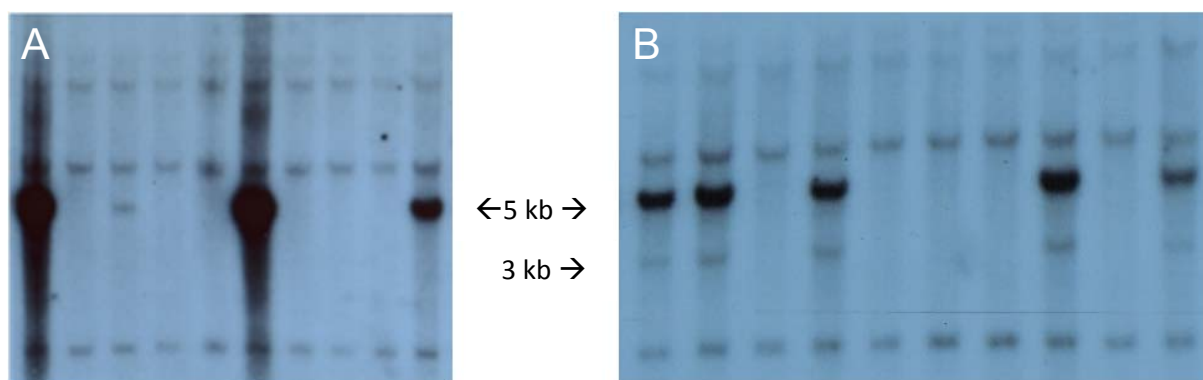
The Southern blot procedure used a radioactively marked probe that hybridized to a 1800 bp region spanning from bases 3 – 1803 of the *Dnmt1s* sequence. This provided a sensitive method to analyze the transgenic status of mice. To ensure that the restriction digest of the genomic DNA used for the Southern blot was not inhibited due to contaminations of the DNA preparation, the agarose gel used for separation of the genomic DNA for the Southern blot was controlled on a UV transilluminator (Fig. 20). After blotting, the *Dnmt1s* region-specific radioactively labelled DNA probe was hybridized to the membrane. The transgenic status was indicated by one or more signals additional to those produced by the endogenous *Dnmt1s* (Fig. 21). The intensity of the Southern blot signal allowed a broad estimate of the number of transgene copies inserted during pronucleus injections (Fig. 21 A). Due to the random integration of the transgene during pronucleus injections, each mouse line had its own specific pattern in the Southern blot, making it possible to distinguish between the different transgenic lines by comparing the signal patterns in the Southern blot, and to determine the transgenic status. Figure 21 A shows a Southern blot from line 1, which has one transgene-specific signal of about 5 kb, whereas Southern blots with DNA from line 4 (Fig. 21 B), produced two transgene-specific signals, one strong signal at about 5 kb, and a weaker, smaller signal at about 3 kb.



**Figure 20. Control of restriction digests for Southern blot**

The successful digest of genomic DNA from mouse tail biopsies was controlled by UV transillumination at  $\lambda=312$  nm of the gels before the Southern blot procedure. Successful digest was indicated by a smear of DNA ranging from ~ 20 kb to ~100 bp. 1<sup>st</sup> lane: 1 kb ladder





**Figure 21. Southern blot comparison of transgenic lines**

Southern blot of offspring from line 1 (A) and line 4 (B). The Southern blot showed four weak signals for the endogenous *Dnmt1s* in all samples. Transgenic mice showed one (A) or more (B) additional signals, with varying strength, depending on the number of sequential insertions of the transgene. Signals from different mouse lines made it possible to distinguish the lines by their specific patterns (one 5 kb signal for line 1, and two signals for line 4, one strong 5 kb signal and one weak 3 kb signal).

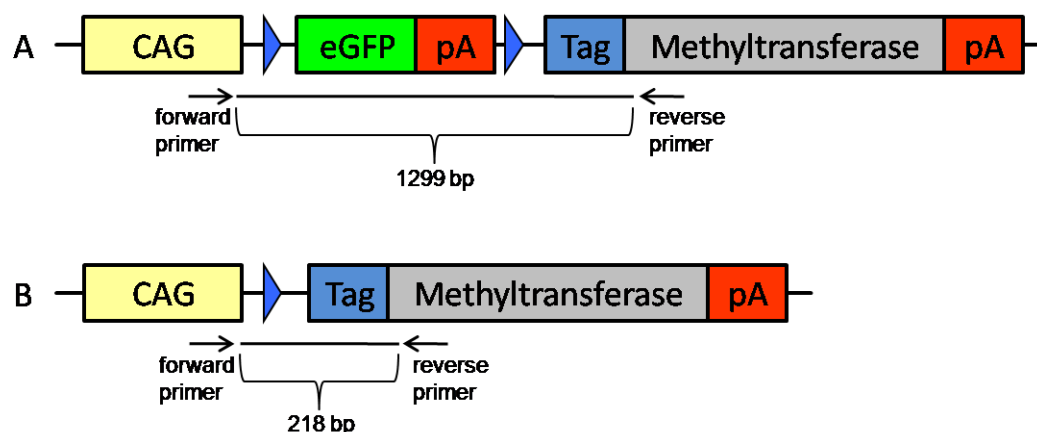
### 3.7 Successful Transgene Recombination after Crossing in with CMV-Cre-recombinant Mouse Strains

Transgenic mice carrying the non-recombined version of the *Dnmt1s* transgene were crossed in with a CMV-Cre recombinant mouse strain, a strain that ubiquitously expresses the Cre-recombinase under the control of the CMV promoter, to investigate the ubiquitous overexpression of *Dnmt1s* from the transgene. The offspring from this cross-in exhibited mendelian inheritance of both the *Dnmt1s* and the Cre-recombinase transgenes, and dams showed no signs of carrying unborn dead embryos. Genomic DNA from the offspring of these cross-ins was analyzed by PCR to determine the recombination status of the transgene, and by Southern blot (as in 3.6) to verify the transgenic status of the mice.

#### 3.7.1 PCR Verification of Transgene Recombination

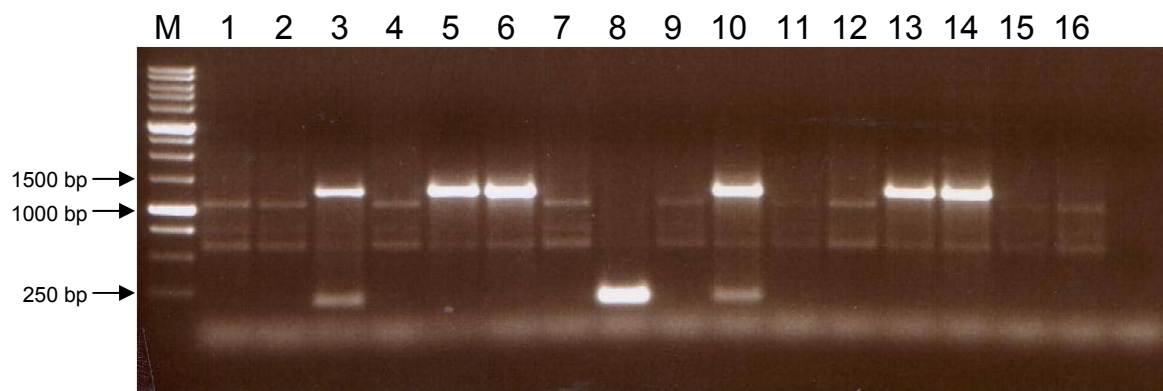
The PCR for the verification of transgene recombination was performed using a forward primer situated in the CAG promoter sequence and a reverse primer situated in the beginning of the *Dnmt1s* cassette (Fig. 22, primer sequences in appendix). By

using this selection of primers, different product sizes were amplified from non-recombined and recombined versions of the transgene. DNA from mice carrying the non-recombined version of the transgene bore a product that spanned from the CAG promoter over the *eGFP* cassette to the beginning of the *Dnmt1s* sequence, resulting in a product size of 1299 bp (Fig. 23, lanes 5, 6, 13 and 14). DNA from mice carrying only the recombined version of the transgene bore a product lacking the *eGFP* sequence due to the excision of the *eGFP* cassette during recombination, resulting in a smaller product of 218 bp (Fig. 23, lane 8). As multiple integrations of the transgene could occur in sequence, it was possible that not all of the copies of the transgene had recombined upon Cre cross-ins, and that some mice might carry both the non-recombined as well as the recombined version of the transgene. PCR amplification of DNA from those mice resulted in both the large 1299 bp fragment and the small 218 bp fragment (Fig. 23, lanes 3 and 10). The remainder of non-recombined transgene copies was avoided in further breeding by choosing mice that exclusively had the small band in PCR analysis.



**Figure 22. Diagram of transgene with primer positions**

The primers used for the PCR to verify the transgene recombination resulted in a 1299 bp fragment from the non-recombined transgene (A), and in a 218 bp fragment from the recombined transgene (B). (figure not drawn to scale)



**Figure 23. PCR for transgene recombination**

DNA from mice carrying one or more copies of the non-recombined version of the transgene produced a 1299 bp product (lanes 5, 6, 13 and 14). DNA from mice carrying one or more copies of the recombined version of the transgene produced a 218 bp fragment (lane 8). DNA from mice carrying multiple transgene insertions of which one or more are non-recombined and one or more are recombined produced both the 1299 bp fragment and the 218 bp fragment (lanes 3 and 10). M: Marker (1 kb ladder)

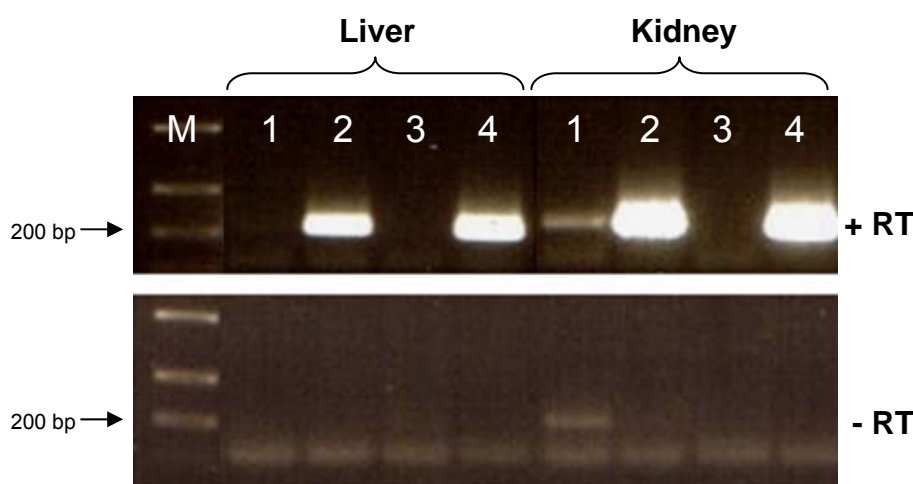
### 3.8 Expression Analyses of Recombined Transgenic Offspring

Mice exclusively carrying recombined copies of the *Dnmt1s* transgene according to the recombination PCR (3.7) were picked for further breeding. They were first bred into a C57BL/6 background to eliminate the presence of the Cre-recombinase. Transgenic offspring of *Dnmt1s*<sup>+</sup>/*Cre*<sup>-</sup> mice were then analyzed for *Dnmt1s* expression in comparison to wild type controls. In this way the influence of the Cre-recombinase on the results of the analyses could be excluded.

#### 3.8.1 The Recombined Transgene Expression of RGS-His-tagged *Dnmt1s* mRNA

Reverse transcriptase PCR (RT-PCR) analysis was performed to determine if the transgene expressed RGS-His-tagged *Dnmt1s* mRNA. To avoid detection of endogenous *Dnmt1s* mRNA, the forward primer for the RT-PCR was placed in the RGS-His-tag sequence, allowing only the specific amplification a 209 bp tagged *Dnmt1s* transgene sequence in combination with the reverse primer (primer

sequences in appendix). Because the *Dnmt1s* sequence of in this transgene is intronless, the cDNA obtained from the reverse transcription has exactly the same sequence as the *Dnmt1s* cassette in genomic DNA of transgenic animals. Therefore, in this study special caution had to be taken to make sure that RNA preparations from transgenic mice were free of genomic DNA contaminations, as both reverse transcribed cDNA as well as genomic DNA yield a fragment in the following PCR. Figure 24 shows an RT-PCR from the liver and kidney of four mice, two each from lines 2 and 4 (+RT), with the corresponding control reaction without reverse transcriptase (-RT). A RNA preparation contaminated with genomic DNA from a mouse that carried a non-recombined copy of the *Dnmt1s* transgene and did not express the transgenic *Dnmt1s* mRNA was included as an example of a false-positive RT-PCR (Fig. 24, kidney, lane 1). The RT-PCR was repeated with all samples that showed contamination with genomic DNA in the -RT control after performing an additional DNase I digest.

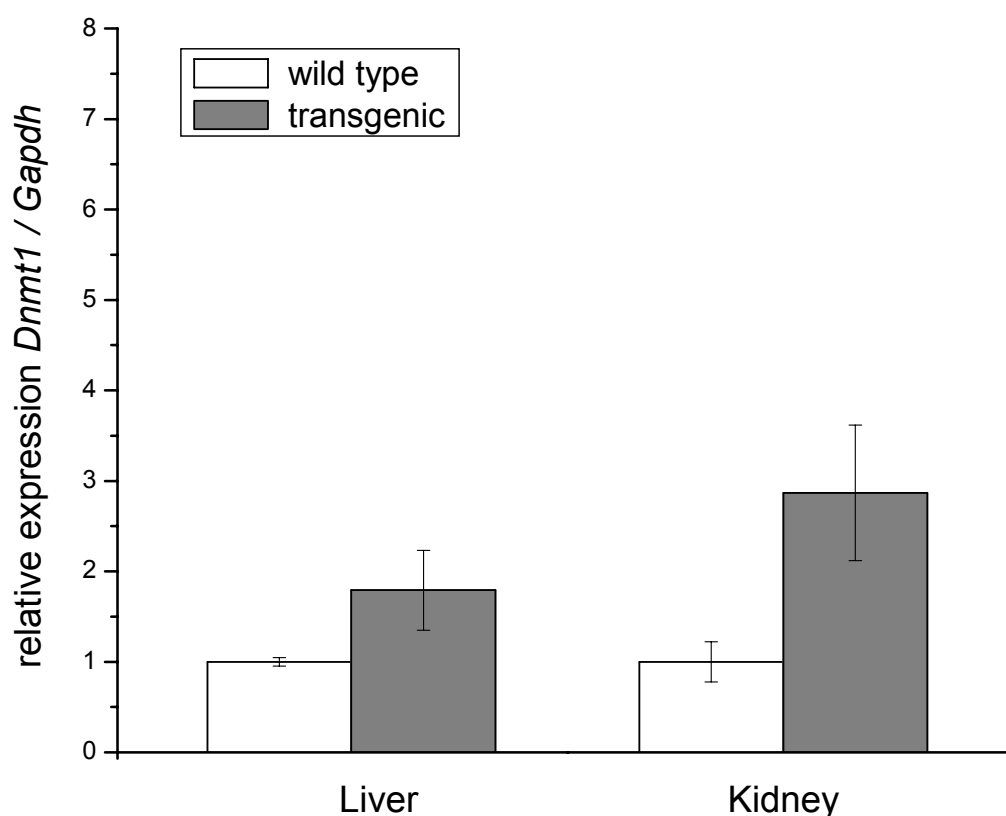


**Figure 24. RT-PCR for verification transgene-specific mRNA**

RT-PCR of liver and kidney RNA of two mice from line 2 (samples 1 and 2) and two mice from line 4 (samples 3 and 4). RGS-His-tagged *Dnmt1s* mRNA was specifically expressed in liver and kidney of mice carrying the recombined version of the transgene, indicated by a 209 bp band (samples 2 and 4). Wild type mice did not express the transgenic mRNA (sample 3). RNA preparations from mice carrying a non-recombined copy of the *Dnmt1s* transgene that were free from genomic DNA contamination also did not yield a product in this RT-PCR (liver, lane 1). However, the presence of a genomic DNA contamination, as in the kidney sample of mouse 1, led to a false-positive 209 bp band in the RT-PCR, but also to a 209 bp band in the -RT reaction (kidney, lane 1). Genomic DNA contamination of RNA preparations was controlled by the -RT results. +RT: complete RT reaction; -RT: control reaction without reverse transcriptase; M: Marker (FastRuler Low Range Ladder)

### 3.8.2 Overexpression of *Dnmt1s* mRNA Induced by the Recombined Transgene

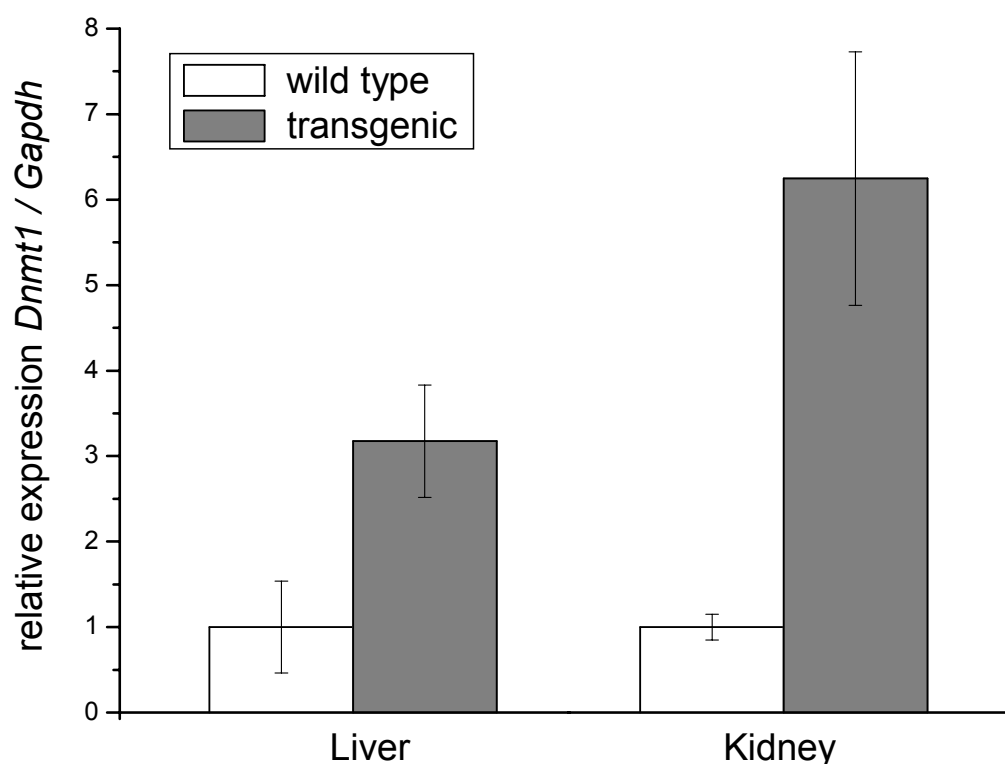
To investigate whether the expression of the transgenic *Dnmt1s* mRNA leads to an overexpression of *Dnmt1s* in total, the combined expression of endogenous and transgenic *Dnmt1s* mRNA from transgenic and sibling wild type mice was compared, using the ABI TaqMan assay for *Dnmt1s* with *Gapdh* as endogenous control. Compared to sibling wild type controls, the assay showed a significant overexpression of total *Dnmt1s* in the liver and kidney of transgenic lines 2 and line 4 (Fig. 25 and 26), but not in the spleen of the same lines (data not shown). Specifically, transgenic animals from line 2 showed a ~2-fold ( $1.79 \pm 0.45$ ) *Dnmt1s* overexpression in liver and a ~3-fold ( $2.86 \pm 0.74$ ) *Dnmt1s* overexpression in kidney compared to sibling wild type controls (Fig. 25).



**Figure 25. TaqMan analysis of *Dnmt1s* expression in liver and kidney of line 2**

Quantitation of *Dnmt1s* expression in liver and kidney of line 2 normalized to *Gapdh* expression using real-time PCR. The relative expression in tissue from transgenic mice was significantly higher than in wild type controls. ( $n \geq 7$ ;  $P < 0.01$ ,  $t$ -test)

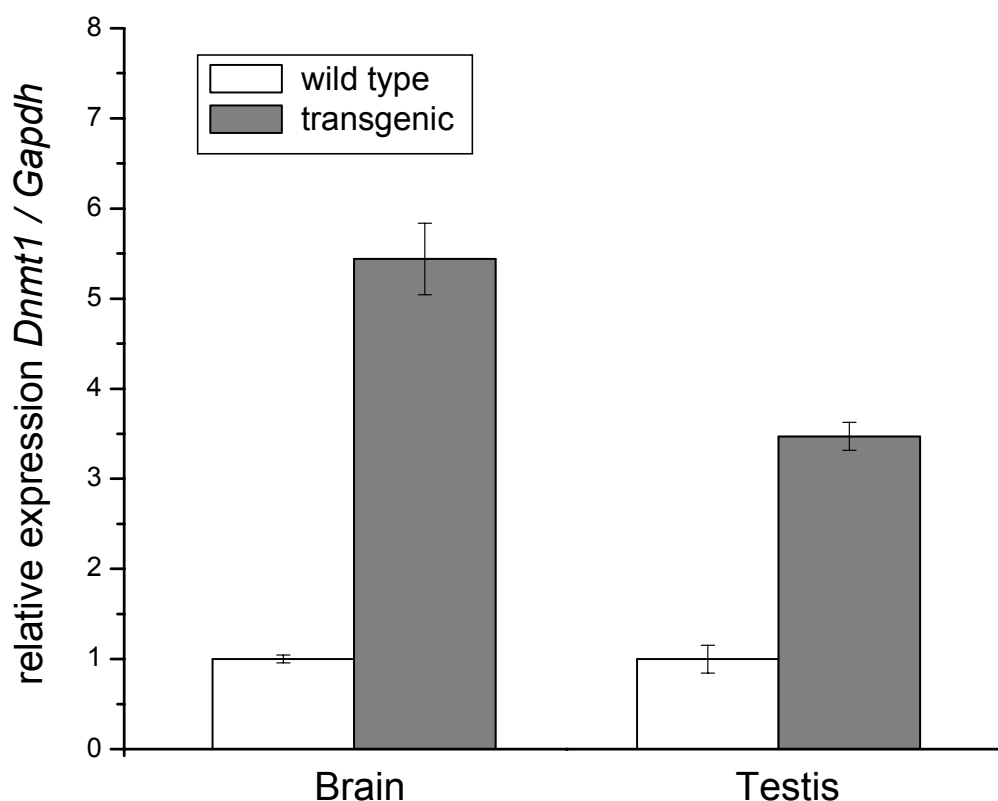
Transgenic mice from line 4 showed a ~ 3-fold ( $3.17 \pm 0.65$ ) *Dnmt1s* overexpression in liver, and a ~6-fold ( $6.24 \pm 1.48$ ) *Dnmt1s* overexpression in kidney compared to sibling wild type controls (Fig. 26). No significant overexpression of *Dnmt1s* could be found in the spleen, liver and kidney of transgenic animals from lines 1, 3 and 5 (data not shown). Transgenic animals from line 6 could not be analyzed due to the overall low birth-rate of transgenic and wild type offspring in this line.



**Figure 26. TaqMan analysis of *Dnmt1s* expression in liver and kidney of line 4**

Quantitation of *Dnmt1s* expression in liver and kidney of line 4 normalized to *Gapdh* expression using real-time PCR. The relative expression in tissue from transgenic mice was significantly higher than in wild type controls. ( $n \geq 8$ ,  $P < 0.001$ , *t*-test)

Because the overexpression of *Dnmt1s* was the highest in transgenic animals from line 4, this line was chosen for TaqMan analysis of *Dnmt1s* expression in two further tissues: brain and testis. TaqMan analysis of the combined expression of endogenous and transgenic *Dnmt1s* mRNA in brain and testis of animals from line 4 showed a ~5.5-fold ( $5.44 \pm 0.39$ ) *Dnmt1s* overexpression in brain and a ~3.5-fold ( $3.47 \pm 0.15$ ) *Dnmt1s* overexpression in testis compared to sibling wild type controls (Fig. 27).



**Figure 27. TaqMan analysis of *Dnmt1s* expression in brain and testis of line 4**

Quantitation of *Dnmt1s* expression in brain and testis of line 4 normalized to *Gapdh* expression using real-time PCR. The relative expression in tissue from transgenic mice was significantly higher than in wild type controls. ( $n \geq 5$ ,  $P < 0.001$ ,  $t$ -test)

### 3.8.3 Overexpression of Dnmt1s Protein Induced by the Recombined Transgene

Because a significant overexpression of *Dnmt1s* on mRNA level caused by the integrated transgene could clearly be shown by TaqMan analyses, tests were carried out to find out whether the transgenic mRNA was translated into protein. To prove the specific expression of the transgenic protein, different methods were attempted, including western blots with an RGS-His-tag specific antibody, purification of the tagged Dnmt1s using a variety of Ni-NTA resins that specifically bind His-tagged proteins, and protein immunoprecipitations using the RGS-His-tag antibody, followed by western blots with a Dnmt1 antibody, or vice versa.

Unfortunately, none of these procedures resulted in reproducible results, so that the translation of the transgenic Dnmt1s protein *in vivo* using the RGS-His-tag sequence could not be verified. However, an indication of the level of Dnmt1s expression can be obtained if a western blot with equal amounts of whole cell extracts is performed and the amount of Dnmt1s protein in the samples using a Dnmt1-specific antibody is compared to the amount of endogenous  $\beta$ -actin protein using a  $\beta$ -actin specific antibody.

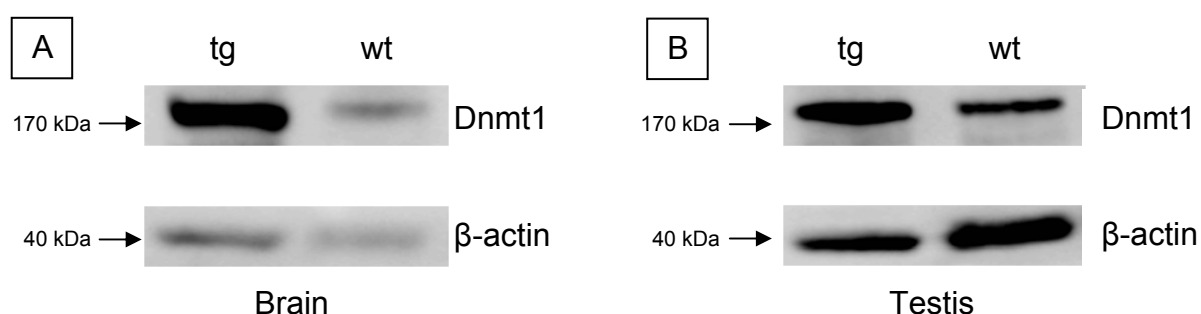
Due to the normalization to the endogenous protein, in this study the amount of the Dnmt1s protein could be compared for transgenic and wild type samples. This method did not specifically prove the expression of the tagged transgenic protein, as it was not specific for the transgenic RGS-His-tagged Dnmt1s protein, but it confirmed that the elevated expression of *Dnmt1s* mRNA also resulted in an elevated expression of the Dnmt1s protein.

To compare the amount of the Dnmt1s protein in transgenic and wild type mice, the following steps were taken: western blots containing 40  $\mu$ g of whole cell extract protein from the brain and testis of transgenic animals and the same amount from sibling wild type samples were prepared. The western blot membrane was then cut in half horizontally at the marker height of 70 kDa. The upper half was then probed with a horseradish-peroxidase conjugated Dnmt1-specific antibody. The lower half of the blot was probed with a horseradish-peroxidase conjugated  $\beta$ -actin antibody. Using the endogenous  $\beta$ -actin signal ( $\sim$  40 kDa) as a control for normalization, the intensity of the Dnmt1s-specific bands ( $\sim$  172 kDa) could be compared between wild



type and transgenic samples. This procedure was performed for the tissues of line 4 that showed the highest expression of Dnmt1s according to the TaqMan analysis, namely kidney, brain and testis. Dnmt1s antibody binding was reproducible for brain and testis samples, but not for kidney samples.

The quantitative western blot analysis of brain and testis protein from line 4 showed a significant overexpression of the Dnmt1s protein in both tissues of transgenic animals compared to sibling wild type controls (Fig. 28). Quantitation calculated a ~ 3-fold Dnmt1s overexpression in brain of transgenic mice and a ~ 6-fold Dnmt1s overexpression in testis of transgenic mice in comparison to sibling wild type controls (Fig. 28).

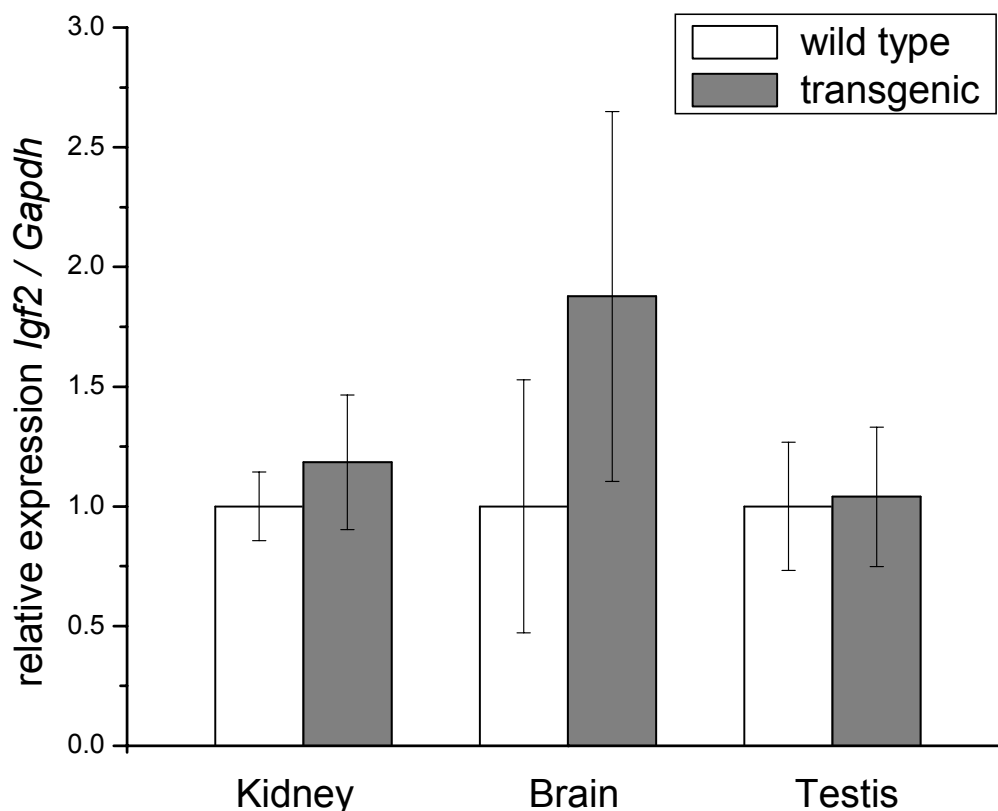


**Figure 28. Western blot quantitation of Dnmt1s expression in brain and testis of line 4**

(A) Quantitation of Dnmt1s protein from brain of line 4. Comparison of Dnmt1s expression normalized to  $\beta$ -actin expression showed a ~3-fold overexpression of Dnmt1s in the brain of transgenic mice. (B) Quantitation of Dnmt1s protein from testis of line 4. Comparison of Dnmt1s expression normalized to  $\beta$ -actin expression showed a ~6-fold overexpression of Dnmt1s in the testis of transgenic mice. Quantitations were performed using the LAS-3000 detection system (Fuji) in combination with the Fuji Multi Gauge software (V3.0) tg: transgenic; wt: wild type.

### 3.9 Influence of *Dnmt1s* Overexpression on *Igf2* Expression *In-vivo*

To analyze whether the overexpression of *Dnmt1s* caused a loss of imprinting and a resulting increase of *Igf2* expression, TaqMan analyses for *Igf2* with *Gapdh* as endogenous control were performed. In the case of the quantitative western blot, the three tissues of line 4 that showed the highest overexpression of *Dnmt1s* according to TaqMan analysis (in 3.7) were chosen for this procedure. The *Igf2* expression in kidney ( $1.18 \pm 0.28$ ), brain ( $1.87 \pm 0.77$ ) and testis ( $1.03 \pm 0.29$ ) of transgenic mice showed no significant difference compared to sibling wild type controls (Fig. 29). This analysis could not confirm an influence of the overexpression of the somatic isoform of *Dnmt1s* on the expression of *Igf2*.



**Figure 29. TaqMan analysis of *Igf2* expression in kidney, brain and testis of line 4**

Quantitation of *Igf2* expression in kidney, brain and testis of line 4 normalized to *Gapdh* expression using real-time PCR. The relative expression in tissue from transgenic mice showed no significant difference to wild type controls in all three tissues. ( $n \geq 5$ ,  $P < 0.05$ ,  $t$ -test)

## 4. Discussion

This study focuses on a mouse model which conditionally overexpresses methyltransferases. But because the overexpression of *Dnmt1* had previously been shown to be embryonically lethal (Biniszkiewicz *et al.* 2002), a conditional approach using the Cre-loxP mechanism was selected and transgene constructs for the somatic isoform of the maintenance DNA methyltransferase *Dnmt1*, *Dnmt1s*, and for the histone methyltransferase *G9A* were prepared.

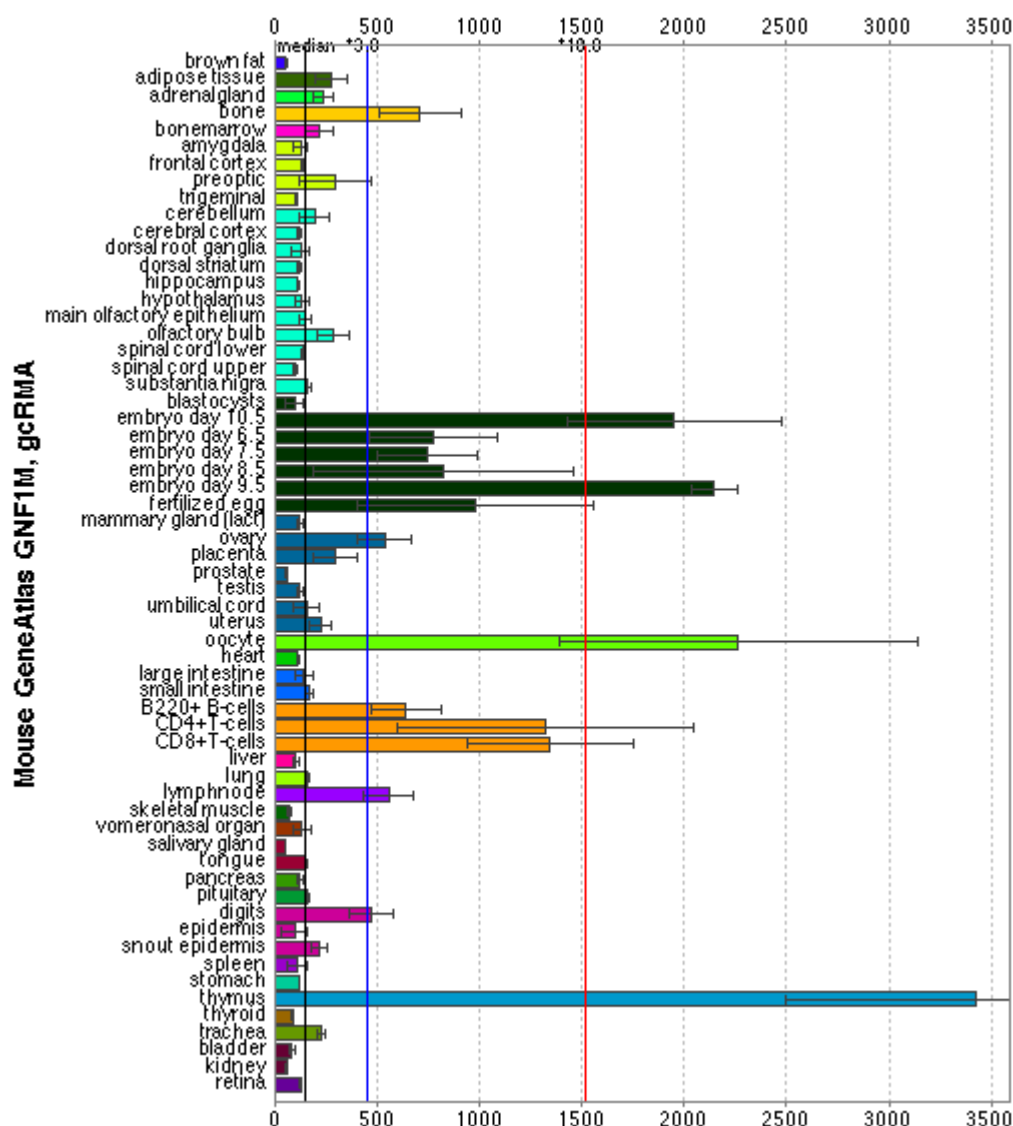
The model system was first established with the *Dnmt1s* transgene before the initiation of a *G9A* transgenic line, so this thesis focused on the creation of a mouse model to study the consequences of *Dnmt1s* overexpression and its effect on the overexpression on the expression of *Igf2*. This enabled verification of the results of the ubiquitous *Dnmt1* overexpression in mice (Biniszkiewicz *et al.*, 2002). Building on the research of this and other previous publications, the gain-of-function *Dnmt1s* mouse model which was created in this study, in combination with the vast spectrum of available Cre-recombinant mouse lines, may well provide a powerful tool to determine whether an overexpression of *Dnmt1s* alone is sufficient to trigger hypermethylation of susceptible parts of the genome in specific tissues or during specific periods of mouse development. It is also a valuable addition to the *in vitro* studies on *Dnmt1* overexpression, which until now have mainly been performed with respect to cancer development (Issa *et al.*, 1993; Wu *et al.*, 1993; Vertino *et al.*, 1996; Ahluwalia *et al.*, 2001).

### 4.1 Ubiquitous *Dnmt1s* Overexpression Levels are Mouse Line- and Tissue-Dependent

Each of the six transgenic mouse lines in this study carried the same transgene for *Dnmt1s* overexpression. TaqMan analysis demonstrated that there were strain-specific variances in *Dnmt1s* transgene expression. Three of the transgenic lines showed no significant overexpression of *Dnmt1* mRNA relative to the endogenous *Gapdh*, while different levels of significant *Dnmt1* mRNA overexpression control were observed in transgenic lines 2 and 4 in this study. The sixth line could not be

analyzed due to an overall low birth rate, although it did exhibit mendelian inheritance of the *Dnmt1s* transgene.

In detail, TaqMan analysis of lines 2 and 4 demonstrated that the transgene did not induce a significant *Dnmt1* mRNA overexpression in the spleen. Line 2 showed low (but significant) levels of *Dnmt1* mRNA overexpression in liver (~2-fold) and kidney (~3-fold). Line 4 significantly overexpressed *Dnmt1* mRNA in rising amounts in the liver (~3-fold), testis (~3.5-fold), brain (~5.5-fold) and the highest amounts in kidney (~6-fold). According to the GeneAtlas of the Genomics institute of the Novartis Research Foundation (GNF) the transcription levels of the endogenous *Dnmt1* are from lowest to highest in kidney, liver, spleen and testis (Fig. 30; Su *et al.*, 2002).



**Figure 30: Tissue dependent mRNA expression of endogenous *Dnmt1***

Black bar: median expression (me); blue bar: me x 3; red bar: me x 10  
(© GNF; source: <http://symatlas.gnf.org/deprecated/>; Su *et al.*, 2002)

Thus, a correlation between the tissue-dependent expression of the transgenic *Dnmt1s* and the endogenous *Dnmt1* could not be found. However, the transgenic *Dnmt1s* expression in brain could not be compared to the GNF GeneAtlas expression profiles, as homogenized whole brain preparations were analyzed in this study, whereas specific areas of the brain were used to analyze the expression in the GNF GeneAtlas. Quantitative western blot analysis of protein expression in brain and testis of animals from line 4 showed ~ 3-fold overexpression of *Dnmt1s* in brain and ~ 6-fold overexpression of *Dnmt1s* in testis in comparison to  $\beta$ -actin expression. These results confirm transgene overexpression on the protein level, but also stand in contrast to the tissue-dependent levels of mRNA expression in TaqMan analyses, which showed higher expression in brain than in testis of mice from line 4.

The observed tissue-dependent variances in transgene overexpression must therefore be taken with caution, as they may be due to the comparison of the *Dnmt1s* transgene expression to endogenous genes that are regulated in a tissue-specific manner. As influences of Cre-recombinase expression could be excluded due to elimination of the Cre-recombinase gene in recombined transgenic animals by crossing back into a neutral C57BL/6 background, the varying degrees of transgene expression are likely to be caused by the transgenic approach itself.

One reason for possible tissue-dependent expression levels could be that the expression of the CMV early enhancer/chicken  $\beta$ -actin (CAG) promoter used in the transgene in this study was ubiquitous, but the expression was controlled to different degrees depending on the type of tissue. This theory is supported by a wide range of publications using pronucleus injections for the creation of animals that carry a transgene with the CAG promoter. The ubiquitous expression of *eGFP* by the CAG promoter has been confirmed in studies with transgenic mice (Okabe *et al.*, 1997) as well as transgenic rabbits (Takahashi *et al.*, 2007), but transgene expression was not quantified in these studies. A second study in transgenic mice investigating the overexpression of *eGFP* under control of the CAG promoter reported ubiquitous expression in all tissues except erythrocytes and hair, and tissue specific differences in transgene expression, but these were only estimated by fluorescence microscopy, and no quantitation was performed (Kawamoto *et al.*, 2000). Further studies however used the CAG promoter to drive a variety of transgenes in mice, and showed ubiquitous but tissue-dependent transgene expression in liver, kidney, spleen, brain,

lung, heart, muscle, dermis, epidermis, testis and oocytes (Araki *et al.*, 1995; Sawicki *et al.*, 1998; Ikeguchi *et al.*, 2004; Wang *et al.*, 2004). A study in rats also confirmed that CAG transgene expression is tissue-dependent in other species (Takeuchi *et al.*, 2003).

A possible mechanism that would explain this differential expression could involve the CAG promoter. Although the CAG promoter is not endogenous to the mouse and shares no significant sequence homology with the mouse genomic reference sequence, it may still be regulated by silencer or enhancer elements that are present in the mouse cells. Consequently, the endogenous regulatory elements may also influence the expression of the transgene, leading to the tissue-dependent differences in transgene expression relative to the endogenous *Gapdh* control observed in this study.

A more feasible explanation for the differences in overall and tissue-dependent expression of the transgenic lines in this study, however, is that the site of integration cannot be controlled using the pronucleus injection technique. The integration site may play a critical role in the overall and tissue-dependent degree of CAG transgene expression (and most likely that of transgenes using other promoters as well), because the expression of endogenous genes is controlled, among others, by the condensation of chromatin. If the transgene is inserted into a chromosomal site that is silenced by chromatin condensation in some or all tissues, it will not be expressed in those tissues: a theory that is consistent with the aforementioned publications. This is furthermore strongly supported by a study where transgenic mice using the CAG promoter to drive the poliovirus receptor were produced by pronucleus injections of a transgene, which showed that tissue-dependent expression can even vary between transgenic lines carrying identical versions of a CAG transgene (Ida-Hosonuma *et al.*, 2002). This would also explain why no significant *Dnmt1s* overexpression could be observed in three of the six transgenic lines produced in this study, while two others showed significant *Dnmt1s* overexpression of varying degrees in most of the analyzed tissues. To furthermore increase the overexpression of *Dnmt1s* in these two lines, homozygous cross-ins could be made. An alternative hereto would be to try and increase *Dnmt1s* activity by raising the availability of the SAM precursor nutrients choline or folate, preferably already during embryogenesis, by feeding a supplemented diet during gestation.

## 4.2 Ubiquitously Overexpressing *Dnmt1s* Mice are Viable

The transgenic offspring of mice that ubiquitously express the *Dnmt1s* transgene in this study are viable, and no obvious phenotype resulting from the *Dnmt1s* overexpression could be observed. These findings stand in contrast to the attempt to create a *Dnmt1* transgenic mouse line, where mice with 4-fold protein levels of Dnmt1 had an embryonic lethal phenotype with death occurring at the latest 12.5 days post coitum (Biniszkiewicz *et al.* 2002).

The reason for this difference in results is likely to lie in the various kinds of transgenes used to create the *Dnmt1* overexpressing lines. The transgene used in this study contains the cDNA sequence for the somatic form of the murine *Dnmt1* gene (*Dnmt1s*). The mouse line established by Biniszkiewicz and colleagues was created using a non-linearized BAC clone. BAC clones are produced by digesting genomic DNA and ligating the fragments into the BAC plasmid vector. The *Dnmt1*-BAC used by Biniszkiewicz and colleagues therefore contained the whole genomic DNA region of the murine *Dnmt1* gene plus 100 kb of upstream genomic DNA, which contains endogenous genes for the intercellular adhesion molecules 1, 4 and 5 (*Icam1*, *Icam4*, *Icam5*), the mitochondrial ribosomal protein L4 (*Mrpl4*) and the sphingosine-1-phosphate receptor 2 (*S1pr2*). Furthermore, because the clone was not linearized prior to transfection, it also contained the BAC plasmid vector backbone sequences. One cannot exclude that the accompanying overexpression of the other endogenous genes introduced with the *Dnmt1* region, or the BAC plasmid vector backbone sequences introduced with the transgene, may have contributed to, or even caused, the phenotypic events observed by Biniszkiewicz and colleagues. Furthermore, because the *Dnmt1*-BAC mouse line contained the genomic DNA of *Dnmt1*, it not only had the capability to overexpress *Dnmt1s*, but also the oocyte-specific and pachytene sperm-specific isoforms of *Dnmt1*, *Dnmt1o* and *Dnmt1p*, respectively. The embryonic lethal phenotype observed by Biniszkiewicz and colleagues therefore may also have resulted from an overexpression of the *Dnmt1o* isoform that is not present in the transgene in this study.

The *Dnmt1o* isoform is translated and enzymatically active during the erasure and re-establishment of DNA methylation marks in the first stages of development of the fertilized oocyte (Carlson *et al.*, 1992; Mertineit *et al.*, 1998). The involvement of

*Dnmt1p* in the embryonic lethal phenotype is less likely, as *Dnmt1p* has not been shown to be expressed on the protein level so far (Trasler *et al.*, 1992; Mertineit *et al.*, 1998; Bestor, 2000). However, a causal relationship between *Dnmt1p* and the embryonic lethal phenotype cannot be excluded, as an involvement of *Dnmt1p* in the regulation of DNA methylation may be present on the RNA level, for example by interference of the multiple short open reading frames of *Dnmt1p* with translation of the open reading frames of *Dnmt1o* or *Dnmt1s* (Bestor, 2000).

Although the timeframe in which the oocyte- and pachytene sperm-specific isoforms are transcribed is relatively small, it is nevertheless a point in development that is essential for the maintenance of DNA methylation of all areas of the genome, including imprinted regions (Bell and Felsenfeld, 2000; Lucifero *et al.*, 2007). The demonstrated hypermethylation of the methylation susceptible retroviral intracisternal A particle (IAP) elements and the *H19* DMD in the *Dnmt1* overexpressing mice created by Biniszkievicz and colleagues is therefore likely to have been caused by an excess of the *Dnmt1o* and/or *Dnmt1p* isoforms during the re-establishment of DNA-methylation.

In combination with this study, it is possible that an overexpression of *Dnmt1o* in the first stages of fertilized oocyte development causes errors in the re-establishment of DNA-methylation, but the overexpression of *Dnmt1s* does not. The corresponding *in vivo* loss-of-function studies confirms this sensitivity to *Dnmt1o* expression levels, as they have shown that *Dnmt1o* deficient mice fail to establish correct methylation patterns during embryogenesis (Cirio *et al.*, 2008 b; Toppings *et al.*, 2008).

Studies with heterozygous *Dnmt1s* knock-out mice revealed hypomethylation of T-lymphocytes DNA in young mice that was compensated with age, suggesting compensatory effects in *Dnmt1s* expression from the remaining allele (Yung *et al.*, 2001; Ray *et al.*, 2006). A recent study using siRNA-mediated knock-down of *Dnmt1s* has, however, suggested that *Dnmt1s* deficiency can lead to growth retardation, but this finding should be taken with caution as it may also be possible that the effect of injected siRNA was retained after implantation to affect the growth thereafter, as the authors themselves stated (Kurihara *et al.*, 2008). Taken together, the results to date may show that the re-establishment of DNA-methylation in the fertilized oocyte might only be sensitive to expression levels of *Dnmt1o*, but not to expression levels of *Dnmt1s*, which would explain the embryonic lethal phenotype of mice in the study by



Biniszkiewicz and colleagues in contrast to the viable mouse model created in this study. To confirm this theory, the overexpression of the *Dnmt1s* transgene during the first developmental stages of the fertilized oocyte would have to be proven.

The theory that the fertilized oocyte might only be sensitive to expression levels of certain DNMTs is consistent with the results of mouse lines for the overexpression of the *de novo* DNA-methyltransferases *Dnmt3a* and *Dnmt3b1* published in 2007 (Linhart *et al.* 2007). Like the transgenic line for overexpression of *Dnmt1s* in this study, neither the *Dnmt3a* nor the *Dnmt3b1* overexpressing transgenic lines had an observable phenotype. *Dnmt3a* has been shown to be required for methylation of imprinted loci during gametogenesis (Kaneda *et al.*, 2004), but even after crossing in with the  $APC^{Min/+}$  mice, a background that is susceptible to colon cancer, the transgenic mouse model for overexpression of *Dnmt3a* showed a normal phenotype. Effects of *Dnmt3b1* overexpression could be shown after the mouse line was crossed in with  $APC^{Min/+}$  mice, but these were long-term effects as they took place during adult development, and could not be shown during embryonic development. *Dnmt3b1* is not required for methylation of imprinted loci during gametogenesis (Kaneda *et al.*, 2004), providing a possible explanation for the *Dnmt3b1/ APC^{Min/+}* mouse model not showing effects in the early embryonic stages, as observed in the *Dnmt1* model from Biniszkiewicz and colleagues. The lack of a phenotype in the *Dnmt3a/ APC^{Min/+}* mice again points to a sensitivity of the fertilized oocytes to which DNMT is overexpressed.

In summary, these findings support the hypothesis that the re-establishment of DNA-methylation of susceptible regions in the fertilized oocyte is sensitive to the overexpression of *Dnmt1o*, but not sensitive to overexpression of *Dnmt3a*, *Dnmt3b1*, and as shown in this study, of *Dnmt1s*. The effects of the *Dnmt1s* overexpression in this mouse model are therefore more likely to have long-term effects on imprinted genes or global methylation, and less likely to cause erroneous imprinting in germ cells or in the first developmental stages of the fertilized oocyte.

### 4.3 Influences of DNMT Overexpression on Gene Expression

The upregulation of imprinted genes encoding for growth factors, such as *Igf2*, has been shown to be present in many different types of tumors. Previous studies have shown that *Igf2* is subject to loss of imprinting which results in biallelic expression and the connected upregulation of *Igf2* in human and murine cancers of the liver, breast, pancreas and colon (Ogawa *et al.*, 1993; Rainier *et al.*, 1993; Steenman *et al.*, 1994; Moulton *et al.*, 1996; Jirtle, 1999). The study of *Dnmt1* overexpression by Biniszkiewicz and colleagues (Biniszkiewicz *et al.*, 2002) as well as the study on *Dnmt3b1* overexpression by Linhart and colleagues (Linhart *et al.*, 2007) showed an influence of DNA methyltransferase overexpression on the expression of *Igf2*. In both cases, the expression of *Igf2* was upregulated as a result of hypermethylation caused by methyltransferase overexpression. However, no direct influence of the overexpression of *Dnmt1s* on the expression of *Igf2* could be confirmed in this study. This difference in results could be explained by the nature of the experiments performed to investigate *Igf2* expression in the two previous studies, and might additionally be explained by the different site preference for *de novo* methylation of *Dnmt1s* and the *Dnmt3* family for the *Dnmt3* overexpression study.

The study by Biniszkiewicz and colleagues investigated the effects of the *Dnmt1* BAC transgene on methylation of the *Igf2/H19* DMD in cell culture experiments, because their mouse model had an embryonic lethal phenotype. The cells used by Biniszkiewicz and colleagues were ES cells that were differentiated into neuronal cells using retinoic acid and leukemia inhibitory factor (LIF) because undifferentiated ES cells do not express *Igf2* and *H19* (Tucker *et al.*, 1996). Due to the nature of the utilized *Dnmt1*-BAC transgene, the overexpression of further endogenous genes introduced with the genomic *Dnmt1* region, or the BAC plasmid vector backbone sequences introduced with the non-linearized transgene, may have contributed to the *Igf2* upregulation observed by Biniszkiewicz and colleagues. It may also be that the *Dnmt1o* and *Dnmt1p* isoforms that are present in the BAC transgene of the study by Biniszkiewicz and colleagues are not only responsible for the epigenetic reprogramming in the first stages of development of the fertilized oocyte, but also for changes in the epigenome during the differentiation of ES cells. In this case, the *Dnmt1* isoforms expressed by the BAC transgene may have led to a methylation of

the *H19* DMD on both alleles during the differentiation of the ES cells, preventing binding of a repressor, the zinc-finger protein CCCTC-binding factor (CTCF), which leads to an upregulation of *Igf2* expression by enhancers downstream of *H19* (Arney, 2003).

The transgenic mouse model in this study does not express the *Dnmt1o* and *Dnmt1p* isoforms, which would explain why changes in *Igf2* expression were observed in the study by Biniszkiwicz and colleagues, but could not be confirmed in this study. A corresponding investigation of differentiating ES cells transfected with the *Dnmt1s* transgene from this study would clarify whether the suggested resistance to *Dnmt1s* overexpression is also present in differentiating ES cells, or whether it is limited to the phase of re-establishment of DNA-methylation in the fertilized oocyte.

In the study by Linhart and colleagues no influence on *Igf2* expression could be observed in mice overexpressing *Dnmt3a*, *Dnmt3b1*, or in cross-ins of *Dnmt3a* and *APC<sup>Min/+</sup>* mice. Cross-ins of the *Dnmt3b1* overexpressing mice with *APC<sup>Min/+</sup>* mice resulted in increased occurrence and size of intestinal adenomas in comparison to *APC<sup>Min/+</sup>* mice that did not overexpress *Dnmt3b1*. The intestinal adenomas and, more importantly, the non-tumor-bearing intestinal mucosa from *Dnmt3b1/ APC<sup>Min/+</sup>* mice both showed a significant overexpression of *Igf2* and a significant down-regulation of *Sfrp2* and *Sfrp5* in comparison to control *APC<sup>Min/+</sup>* mice. Hypermethylation of the *H19* DMD and of the *Sfrp2* and *Sfrp5* promoter regions could also be detected in both normal intestinal mucosa and intestinal adenomas. This shows that a susceptible background is necessary for *Dnmt3b1* overexpression to trigger an increase of *Igf2* expression and a decrease of *Sfrp2* and *Sfrp5* expression caused by aberrant methylation, whereas for *Dnmt3a* overexpression even a susceptible background does not lead to aberrant methylation. The upregulation of *Igf2* expression by *Dnmt3b1* observed in the study by Linhart and colleagues was therefore observed under conditions that were not present in this study. Crossing in the transgenic mouse model for the overexpression of *Dnmt1s* from this study with the colon cancer susceptible *APC<sup>Min/+</sup>* background would clarify whether *Dnmt1s* overexpression could affect gene regulation in adult animals by aberrant methylation like *Dnmt3b1*, or if DNA-methylation in adult animals is resistant to the overexpression of *Dnmt1s*, as in the case of *Dnmt3a*. When performing this proposed analysis, it would be important to consider the different preferential sites of *Dnmt1s* and the *Dnmt3* family for de

*de novo* methylation that were found *in vitro*, as the regions targeted by Dnmt1s *in vivo* may also differ from the regions that were found to be differentially methylated in the *Dnmt3b1* mouse model (Handa and Jeltsch, 2005; Goyal *et al.*, 2006).

In fact, this is a further possible explanation for the difference in the results observed for *Igf2* expression between the *Dnmt3b1* mouse model and the mouse model in this study. Less than 10 % of the CpGs in the *H19* DMD and in the promoter regions of *Sfrp2* and *Sfrp5* are CpGs in the preferential CCGG context of Dnmt1s: 5 of 59 are CCGG in the 2.2 kb region between 2 and 4 kb upstream of the *H19* start of transcription (Tremblay *et al.*, 1997); 7 of 120 are CCGG in the total 5212 bp 5' UTR sequence of *H19*, (GenBank accession no. U19619.1; Tremblay *et al.*, 1995); 11 of 104 are CCGG in the CpG island comprising the *Sfrp2* promoter; 6 of 85 are CCGG in the CpG island comprising the *Sfrp5* promoter. Consequently, the lack of preferred recognition sites for Dnmt1s (or in other words, the abundance of preferred recognition sites for Dnmt3b1) may be the reason why no changes in *Igf2* expression were found in the *Dnmt1s* mouse model in this study, in contrast to the study with *Dnmt3b1* mouse model. This increases the importance of choosing candidate genes that have as many CCGG motifs as possible when investigating cross-ins of APC<sup>Min/+</sup> with the *Dnmt1s* strain produced in this study. The incorporation of the *H19* DMD and the *Sfrp2* and *Sfrp5* promoter region CpG islands would nevertheless be important to verify whether the preferential sites for *de novo* methylation found *in vitro* are also reflected in an *in vivo* situation. An alternative to this approach would be to first analyze the *Dnmt1s* transgenic animals with the anti-Methylcytidin-ChIP (-on-chip) method to determine candidate genes.

In summary, transfection experiments with the *Dnmt1s* transgene would show if differentiating ES cells are susceptible or resistant to Dnmt1s overexpression. Crossing in the *Dnmt1s* mouse line into the APC<sup>Min/+</sup> background would show if somatic cells are susceptible to methylation by Dnmt1s, as in the case of Dnmt3b1, or if they are resistant to Dnmt1s methylation, as in the case of Dnmt3a. The cross-ins would also confirm the different specificities for *de novo* methylation of Dnmt1s and Dnmt3b1 *in vivo*, and might even reveal genes that are targeted for *de novo* methylation by only one of the two methyltransferases.

#### 4.4 Further Characterization of the *Dnmt1s* Overexpressing Mouse Line

In this study a mouse line that conditionally overexpresses *Dnmt1s* was successfully established. Although the RT-PCR results show that the RGS-His-tagged transgenic *Dnmt1s* is specifically transcribed on mRNA level, and leads to a significant increase of total *Dnmt1* mRNA expression, the situation is less clear on the protein level. The translation of the RGS-His-tagged protein could be shown during *in vitro* transgene testing using a combination of cell culture co-transfection and a western blot with an RGS-His-tag specific antibody. However, when various methods were attempted to detect the *in vivo* protein using the RGS-His-tag, none of these procedures resulted in reproducible results, so that a specific verification of the translation of the transgenic *Dnmt1s* mRNA into a protein *in vivo* could not be achieved. A reason for the failure to detect RGS-His-tagged Dnmt1 in transgenic mice may lie in the structure of the transgene in this study. In addition to the ATG translational start site before the RGS-His-tag, there is another ATG site after the tag, at the beginning of the *Dnmt1* open reading frame. The RGS-His-tag is patented by Qiagen, and the “QIAexpress<sup>®</sup> Detection and Assay Handbook” (Qiagen, supplied with RGS-His-tag plasmids) states that “an additional (...) internal start site close to the N-terminus of the insert could result in overexpressed, nearly full-size protein lacking a tag”. Although the RGS-His-tagged Dnmt1 could be detected in cell culture testing of the transgene, the conditions in the mouse model created in this study are different, as the abundance of transgenic DNA is lower in the cells of the live animal than in cell culture transfections. This could explain the problems of detecting RGS-His-tagged Dnmt1 in transgenic mice.

Tests using the quantitative western blot strategy with a Dnmt1s specific antibody, however, did successfully show that the total expression of Dnmt1s protein in animals carrying the recombined version of the transgene is significantly elevated in brain and testis in comparison to sibling wild type control animals. Although this does not specifically prove the translation of the transgenic Dnmt1s, it does strongly suggest that the transgene is also expressed on the protein level, as this is the only feasible explanation for the elevated *Dnmt1s* expression in animals carrying the recombined transgene measured in the quantitative western blot.

Before the *Dnmt1s* mouse model created in this study can be confirmed as fully validated, the functionality of the transgenic protein still needs to be addressed. If a phenotype in form of hypermethylation could be detected in animals carrying the recombined transgene, this would be an indication for the functionality of the transgenic protein. The proposed cross-ins into a background susceptible to methylation may help to find differences in methylation between *Dnmt1s* overexpressing and control animals. Comparing the *in vitro* methylation activity of protein extracts from animals carrying the recombined transgene to sibling wild type control animals might be another way of verifying the functionality of the transgenic protein.

A real proof for the specific functionality of the transgenic protein, however, would be based on testing the *in vitro* methyltransferase activity of an RGS-His-tag purified Dnmt1s protein extract, or a ChIP-Seq or ChIP-on-chip assay on DNA from highly overexpressing tissues of transgenic mice in comparison to sibling wild type controls.

#### **4.5 Focus of Future Research Using the *Dnmt1s* Overexpressing Mouse Line**

The gain-of-function *Dnmt1s* strain created in this study may well be a valuable system for the investigation of the effects of *Dnmt1s* overexpression in several fields of research, as a viable transgenic mouse model for the *in vivo* overexpression of *Dnmt1* has not been reported so far.

*First*, this model could give further insight on the genes that are regulated by DNA methylation, especially in combination with previous overexpression studies on *Dnmt1* (Wu *et al.*, 1993; Vertino *et al.*, 1996; Biniszkiwicz *et al.*, 2002; Feltus *et al.*, 2003). CpG islands associated with promoter regions of genes that have been found to be hypermethylated in these studies such as *E-CAD*, *ER*, *HBA*, *HIC-1*, *H19*, *Igf2*, *SST* or at least predicted to be hypermethylated in these study by Feltus and colleagues (*CDX2*, *GRB10*, *TBR1*, *Ipf1*, *SIM2*, *GRM6*, and *XT3*) would be a good choice for a candidate gene approach. Retroviral elements like Alu-repeats, Tandem B1-repeats or IAPs could also be analyzed for hypermethylation. Consideration of the amount of the reported preferential sequence of Dnmt1 for *de novo* methylation would give insight into the relevance of this *in vivo*. Cross-ins with a colon cancer

susceptible background like the APC<sup>Min/+</sup> model, or feeding the *Dnmt1s* mice with an excess of choline or folate to increase the availability of the methyl-group donor SAM, could increase the chances of finding regions prone to aberrant methylation caused by *Dnmt1s*, and in addition would help to determine if *Dnmt1s* has the same promoting effect on tumorigenesis as found by Linhardt and colleagues for *Dnmt3b1* (Linhardt *et al.*, 2007).

*Second*, it has been shown that Dnmt1 can repress transcription independently of its methyltransferase activity as well as target certain co-repressors to replication foci, showing that Dnmt1 does not only duplicate DNA methylation patterns, but also re-establishes parental states of gene activity on newly replicated DNA (Fuks *et al.*, 2000). The *Dnmt1s* mouse model produced in this study may therefore also be an effective tool to study the co-factors that have been shown to be involved in methylation by Dnmt1, as in the case of the methyl-group donor SAM or the Dnmt1 recruiting proliferating cell nuclear antigen (PCNA) (Adams *et al.*, 1974; Chuang *et al.*, 1997; Malkas, 1998; Iida *et al.*, 2002), and to study the Dnmt1 binding co-repressors, such as DMAP1, E2F1, HDAC1, HDAC2, LSH, MeCP2, MBD2, MBD3, and Rb (Rountree *et al.*, 2000; Tatematsu *et al.*, 2000; Robertson *et al.*, 2000; Pradhan and Kim, 2002; Kimura and Shiota, 2003; Kimura *et al.*, 2003; Liu and Fisher, 2004; Myant and Stancheva, 2008). It would be interesting to investigate if the consequences of *Dnmt1s* overexpression are in some way compensated by the regulation of one or more of these co-factors or co-repressors, and furthermore to analyze which of them are limiting factors in *Dnmt1s* mediated DNA-methylation or transcriptional repression, respectively.

*Third*, the *Dnmt1s* mouse strain also provides a model system to study the consequences of *Dnmt1s* overexpression on other DNMTs. It has been reported that combined action of both *de novo* and maintenance DNA-methyltransferases is required for the maintenance of DNA methylation in adult mammals and the re-establishment of genomic methylation patterns in the fertilized oocyte (Okano *et al.*, 1999; Robertson *et al.*, 1999; Ratnam *et al.*, 2002; Chen and Li, 2006; Lucifero *et al.*, 2007). Furthermore, the knockout of one DNMT can lead to compensation of methylation by the remaining DNMTs (Kim *et al.*, 2002; Ko *et al.*, 2005), and interactions within the mammalian DNMT family itself have been shown (Margot *et al.*, 2003). Analysis of the effect of the overexpression of *Dnmt1s* on the expression

levels of the remaining DNMTs may provide additional information on these mechanisms.

*Fourth*, the *Dnmt1s* mouse model is also suitable for analyzing the regulation of Dnmt1 protein levels. It has only recently been shown that proteasome-mediated degradation of Dnmt1s is regulated by monomethylation of Lys-142 of Dnmt1s by *Set7*, and that *Set7* protein levels directly influence the availability of Dnmt1s in mammalian cells (Estève *et al.*, 2009). In this respect, the analysis of expression and activity of *Set7* in the *Dnmt1s* model would give insight into the question whether *Set7* expression is coupled *in vivo* to the expression or the protein levels of *Dnmt1s*.

*Fifth*, the *Dnmt1s* mouse model could be used to study the effect of *Dnmt1s* overexpression on histone modifications, specifically those mediated by G9A, Suv39h1 and Suv39h2, which have been shown to interact with DNMTs (Fuks, 2003; Estève *et al.*, 2007). The analysis of acetylation of H3K9 and methylation of H3K27 in the *Dnmt1s* mouse model would clarify if this is a way of counteracting excess levels of Dnmt1s *in vivo* besides *Set7* induced degradation. Vice versa, analysis of H3K9 methylation and H3K27 acetylation could provide information on whether the overexpression of Dnmt1s leads to methylation changes that in turn trigger transcriptional silencing by histone modifications. The establishment of a mouse line for the overexpression of G9A, for which a transgene was prepared in this study, will make cross-ins possible that overexpress both *Dnmt1s* and G9A. This combined model would be particularly interesting as it would enable the study of *in vivo* interaction between *Dnmt1s* and G9A, which has been shown to coordinate DNA and histone methylation during replication. This interaction has so far only been demonstrated in *in vitro* cell culture experiments (Estève *et al.*, 2006).

*And finally*, the mouse model in this study may furthermore provide an ideal basis for studying the effects of long-term *Dnmt1s* overexpression on cancer development. Most studies investigating the role of *Dnmt1* methylation in cancer are *in vitro* loss-of-function studies by inhibition of *Dnmt1* (Robert *et al.*, 2003) probably because a homozygous knock-out of *Dnmt1* by mutation leads to *Xist* upregulation by demethylation and an embryonic lethal phenotype (Li *et al.*, 1992; Beard *et al.*, 1995). *In vivo* *Dnmt1s* deficient models using heterozygous knock-out had no effect on longevity, but showed deregulation of *H19*, *Igf2* and *Igf2r* genes, unstable X inactivation and T-lymphocyte DNA hypomethylation (Li *et al.*, 1993; Sado *et al.*,



2000; Yung *et al.*, 2001; Ray *et al.*, 2006). These loss-of-function studies are based on the finding that cancer is associated with global hypomethylation and hypomethylation of (proto-) oncogenes. Another important hallmark of cancer, however, is the hypermethylation of tumor suppressor genes. In this sense, the *Dnmt1s* mouse model may yet provide an *in vivo* model for certain types of cancer. As no long-term effects on cancer development have been observed in the *Dnmt1s* model so far, it may be necessary to cross in the mice with a cancer susceptible background, such as the 129S1/SvImJ (129/Sv) and 129.MOLF-Chr19 strains for testicular cancer (Youngren *et al.*, 2003; Zhu *et al.*, 2007 ), the A/J and SWR/J strains for lung cancer (Manenti *et al.*, 1995; Manenti *et al.*, 1997), the BALB/c strain which is sensitive to ionizing radiation-induced mammary tumors (Yu *et al.*, 2001), or one of the many transgenic strains which are meanwhile available for breast cancer (Radisky *et al.*, 2009).

The mouse model for *Dnmt1s* overexpression will certainly also be useful in further areas of research that have not been addressed here. The establishment and cross-in of the mouse line for conditional overexpression of *G9A*, for which a functional transgene was cloned in this study, could even extend the range of possible applications. Hopefully, some of the many epigenetic mechanisms which have not been understood so far will be clarified with the help of the mouse model for conditional overexpression of *Dnmt1s* created in this study.

## 5. Summary

One of the epigenetic mechanisms by which gene expression is regulated is the methylation of DNA. Erroneous DNA methylation can cause a vast spectrum of diseases. A mouse model was created in this study to investigate whether the overexpression of the somatic form of *Dnmt1*, *Dnmt1s*, is sufficient to cause erroneous methylation and disease.

A CAG promoter-driven transgene containing the *Dnmt1s* DNA methyltransferase was constructed. Transgene functionality was tested in 3T3 murine fibroblasts in cell culture. Because the ubiquitous overexpression of *Dnmt1* has been reported to be embryonically lethal, a conditional transgene was constructed using the Cre-LoxP system. This technique allows the initial expression of the eGFP marker protein only. Upon cross-ins with Cre-recombinant mouse lines, the eGFP sequence is cut out and degraded. This leads to the expression of the *Dnmt1s* under control of the CAG promoter. To easily distinguish between endogenous and transgenic methyltransferase, a RGS-His-tag was added to the N-terminal end of the *Dnmt1s* transgene.

Pronucleus injections with the *Dnmt1s* transgene resulted in six founder lines verified by PCR, Southern blot and eGFP fluorescence. Cross-ins of these founders with CMV-Cre mouse lines yielded viable offspring. The ubiquitous overexpression of *Dnmt1s* was investigated in cross-ins that carry the recombined version of the *Dnmt1s* transgene and express RGS-His-tagged Dnmt1 as verified by RT-PCR. TaqMan analyses of total *Dnmt1* expression of the line with the strongest overexpression showed a ~ 3-fold ( $3.17 \pm 0.65$ ) overexpression in liver, a ~ 3.5-fold ( $3.47 \pm 0.15$ ) overexpression in testis, a ~ 5.5-fold ( $5.44 \pm 0.39$ ) overexpression in brain and a ~ 6-fold ( $6.24 \pm 1.48$ ) overexpression in kidney, but no overexpression in the spleen of CMV-Cre recombined transgenic mice in comparison to sibling wild type controls. Dnmt1 overexpression was confirmed on the protein level by quantitative western blots, which detected ~ 3-fold Dnmt1 levels in brain and ~ 6-fold Dnmt1 levels in testis of transgenic mice in comparison to sibling wild type controls. Upcoming analyses will test the *in vivo* functionality of the transgenic protein and the phenotypic consequences of *Dnmt1s* overexpression.

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## 6. References

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## 7. Appendix

### 7.1 Oligonucleotides (Primers)

#### Primers and thermocycler program for the transgene insertion PCR (2.2.7.5 / 3.6)

##### Dnmt1s transgene - product size: 532 bp

GFP\_US\_1                      5'-CCAACGAGAAGCGCGATCACATG-3'  
 Dnmt1\_rec\_LS1                5'-CCGGGAGCGAGCCTGCCGGGGAG-3'

##### Rag1control fragment - product size: 295 bp

mRag1.1                      5'-GCTGATGGGAAGTCAAGCGAC-3'  
 mRag1.3                      5'-GGGAAGTCTGAACTTTCTGTG-3'

96 °C	2 min	hold
96 °C	25 s	} 35 cycles
56 °C	25 s	
72 °C	2 min	
72 °C	10 min	hold
4 °C	∞	hold

#### Primers for the production of the Southern blot probe (2.2.7.4.1 / 3.6) product size: 1800 bp

Dnmt1\_gU\_S3                5'-GCCAGCGCGAACAGCTCCAGCCC-3'  
 Dnmt1\_g\_LS3                5'-GATAGACCAGCTTGGTGGTGGTGGC-3'

The PCR conditions are described in section 2.2.7.3.1.

#### Primers and thermocycler program for the transgene recombination PCR (2.2.9 / 3.72) product sizes: 218 bp, 1299 bp

CAG\_US\_1                      5'-CTGGTTATTGTGCTGTCTCATC-3'  
 Dnmt1\_rec\_LS1                5'-CCGGGAGCGAGCCTGCCGGGGAG-3'

96 °C	2 min	hold
96 °C	25 s	} 35 cycles
56 °C	25 s	
72 °C	2 min	
72 °C	10 min	hold
4 °C	∞	hold

Primers and thermocycler program for the transgene specific RT-PCR  
(2.2.11.4 / 3.8.1) product size: 209 bp

HIS\_RT\_US2                      5'-TCATCACCATCACCATATGCC-3'  
PRIMER3\_RT\_LS                5'-CCAAGTCACACAACTGGCTTT-3'

96 °C	2 min	hold
96 °C	25 s	} 35 cycles
60 °C	25 s	
72 °C	2 min	
72 °C	10 min	hold
4 °C	∞	hold

Primers and thermocycler program for sequencing templates (2.2.7.3 / 3.4.1)

front fragment - product size: 3164 bp

cDNMT1\_1for                    5'-CTGGTTATTGTGCTGTCTCATC-3'  
Dnmt1\_g\_LS1                  5'-CTTATCATACTTCTCAATCTGC-3'

back fragment - product size: 3299 bp

Dnmt1\_g\_US2                  5'-GCTGGTCTATCAGATCTTTGAC-3'  
cDNMT1\_6405rev              5'-CACAGAAGTAAGGTTCTTCA-3'

96 °C	2 min	hold
96 °C	25 s	} 35 cycles
56 °C	25 s	
72 °C	2 min	
72 °C	10 min	hold
4 °C	∞	hold

Primers for Transgene sequencing (3.4.1)

cDNMT1\_1for                    5'-CTGGTTATTGTGCTGTCTCATC-3'  
cDNMT1\_155for                5'-CTGTTCCACCGGGGTGGTGCC-3'  
cDNMT1\_350for                5'-AGCCGCTACCCCGACCAT-3'  
cDNMT1\_425rev                5'-TGCGCTCCTGGACGTAGCCT-3'  
cDNMT1\_664for                5'-GCAGCTCGCCGACCACTACC-3'  
cDNMT1\_707rev                5'-CGTCGCCGATGGGGGTGTTC-3'  
cDNMT1\_797for                5'-CTGGAGTTCGTGACCGCCGC-3'  
cDNMT1\_946rev                5'-CAGGGGGAGGTGTGGGAGGT-3'

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cDNMT1_1200for	5'-CCCATGGGTAGAGGTTCTCA-3'
cDNMT1_1304rev	5'-ATGGTCCGGGAGCGAGCCTG-3'
cDNMT1_1568for	5'-CCGGCCAACCTGGAGAGCAG-3'
cDNMT1_1644rev	5'-TCCTGGGTCCCCGAGGCTTG-3'
cDNMT1_1792for	5'-CGGCTGAGTCGGCTGCAGAG-3'
cDNMT1_1874rev	5'-CACAGCAGCTGCAGCACCAC-3'
cDNMT1_2073for	5'-TCCAGACCCAGGAGCCAGCC-3'
cDNMT1_2119rev	5'-GGTCTCCGTTTGGCAGCTGGA-3'
cDNMT1_2421for	5'-TCCACCTCGACCTGGTTTGATACT-3'
cDNMT1_2516rev	5'-GACAGGACACAGGTGCCCGC-3'
cDNMT1_2794for	5'-TCCTGCAAAACAATCCTGATGCTGT-3'
cDNMT1_2914rev	5'-TGGGCGTGGCGTAAGAGGGA-3'
cDNMT1_3021for	5'-TCCCTGGGACAGAGGCGAGC-3'
cDNMT1_3323for	5'-GGCGGTGAAGGAGGCAGACG-3'
cDNMT1_3447rev	5'-GCCCCAAGCCAGGAGATGCGG-3'
cDNMT1_3619for	5'-ATGCGCACTGGTTCTGCGCT-3'
cDNMT1_3785rev	5'-GCCTCCCTCCATGGCCCAGT-3'
cDNMT1_3798for	5'-ACCACACTGCCTGGGGCTGA-3'
cDNMT1_4005rev	5'-CCCGGCCATCCACCTCCTCA-3'
cDNMT1_4099for	5'-TGGCTAGCCCCGTGAAACGC-3'
cDNMT1_4414for	5'-AGGGCCGCTGTACCGTGGAG-3'
cDNMT1_4489rev	5'-CGGTCAGGGCCCCCTTGTGA-3'
cDNMT1_4774for	5'-TTCGGCTGAACAACCCCGGC-3'
cDNMT1_4882rev	5'-TGTGGCAGCCTTTGGCCCAG-3'
cDNMT1_5100for	5'-TGCCTGGTCCGCATGGGCTA-3'
cDNMT1_5237for	5'-GCATGTGTTTGCGCCCCGTG-3'
cDNMT1_5338rev	5'-ATGGTTCGGAAGGGCCCCGA-3'
cDNMT1_5679for	5'-GCAGAAGGCAAGGCCTGCGA-3'
cDNMT1_5786rev	5'-CCCGTAGAGGCCAGCCCAGT-3'
cDNMT1_5835for	5'-CCCATGGGCAAGCAGGGTCG-3'
cDNMT1_6025for	5'-GCCTGCTGTCCAGTGCTCGG-3'
cDNMT1_6127rev	5'-CAGTGCACGTGGGGCTCTGG-3'
cDNMT1_6405rev	5'-CACAGAAGTAAGGTTCTTCA-3'
CAG_LS_220	5'-GACGTCAATGGGTGGACTATTTAC-3'
GFP_LS_1	5'-AGGGCGAGGGCGATGCCACCTA-3'
GFP_LS_2	5'-CCCGTGCTGCTGCCCGACAACC-3'
rec_US_100	5'-CAGGTTCAAGGGGAGGTGTGGG-3'
Dnmt1gLS1	5'-CTTATCATACTTCTCAATCTGC-3'
Dnmt1gUS2	5'-GCTGGTCTATCAGATCTTTGAC-3'

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## 7.2 TaqMan Gene Expression Assays (2.2.11.5 / 3.8.2 / 3.9)

<i>Dnmt1</i>	TaqMan Gene Expression Assay, ID: Mm01151063_m1
<i>Igf2</i>	TaqMan Gene Expression Assay, ID: Mm00439564_m1
<i>Gapdh</i>	TaqMan Endogenous Control Part No.: 4352932E (equivalent to Assay ID: Mm99999915_g1).

## 7.3 pLCAG-eGFP-loxP-Dnmt1s Transgene Sequence (2.2.1.8 / 3.4.1)

### Legend:

**Orange:** CAG Promoter

**Light Blue:** LoxP Site

**Green:** eGFP sequence

**Dark Blue:** RGS-His-tag

**Grey:** *Dnmt1s* Sequence

**Black:** others

GGCCGC**GTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGT**  
**CATTAGTTCATAGCCCATATATGGAGTTCGCGTTACATAACTTACGGTAAATG**  
**GCCCGCCTGGCTGACCGCCCAACGACCCCGCCATTGACGTCAATAATGAC**  
**GTATGTTCCCATAGTAACGCCAATAGGGACTTTCATTGACGTCAATGGGTGG**  
**ACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCA**  
**AGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGC**  
**CCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGT**  
**CATCGCTATTACCATGGGTGAGGTGAGCCCCACGTTCTGCTTCACTCTCCCC**  
**ATCTCCCCCCCCCTCCCCACCCCAATTTTGTATTTATTTATTTTAAATTATTTG**  
**TGCAGCGATGGGGGCGGGGGGGGGGGGGGGGGCGCGCGCCAGGCGGGGCGGGC**

GGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAAT  
CAGAGCGGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGC  
GGCCCTATAAAAAGCGAAGCGCGCGGGCGGGCGGGAGTCGCTGCGTTGCCTTC  
GCCCCGTGCCCCGCTCCGCGCCGCTCGCGCCGCCCGCCCCGGCTCTGACTG  
ACGCGTTACTCCACAGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCT  
GTAATTAGCGCTTGGTTTAATGACGGCTCGTTTCTTTTCTGTGGCTGCGTGAAA  
GCCTTAAAGGGCTCCGGGAGGGCCCTTTGTGCGGGGGGGAGCGGCTCGGGG  
GGTTCGTGCGTGTGTGTGTGCGTGGGGAGCGCCGCGTTCGGGCCGCGCTGC  
CCGGCGGCTGTGAGCGCTGCGGGCGCGGCGCGGGGCTTTGTGCGCTCCGCG  
TGTGCGCGAGGGGAGCGCGGCCGGGGGCGGTGCCCGCGGTGCGGGGGGG  
CTGCGAGGGGAACAAAGGCTGCGTTCGGGGGTGTGTGCGTGGGGGGGTGAGC  
AGGGGGTGTGGGCGCGGCGGTTCGGGCTGTAACCCCCCCTGCACCCCCCTC  
CCCGAGTTGCTGAGCACGGCCCCGGCTTCGGGTGCGGGGCTCCGTGCGGGGC  
GTGGCGCGGGGCTCGCCGTGCCGGGCGGGGGGTGGCGGCAGGTGGGGGTG  
CCGGGCGGGGCGGGGCCGCTCGGGCCGGGGAGGGCTCGGGGGAGGGGCG  
CGGCGGCCCGGAGCGCCGGCGGCTGTGAGGCGCGGCGAGCCGCAGCCAT  
TGCTTTTATGGTAATCGTTCGAGAGGGCGCAGGGACTTCCTTTGTCCCAAAT  
CTGGCGGAGCCGAAATCTGGGAGGCGCCGCCGCACCCCCTCTAGCGGGCGC  
GGGCGAAGCGGTGCGGCGCCGGCAGGAAGGAAATGGGCGGGGAGGGCCTT  
CGTTCGTGCGCGCGCCGCGCTCCCCTTCTCCATCTCCAGCCTCGGGGCTGCC  
GCAGGGGGACGGCTGCCTTCGGGGGGGACGGGGCAGGGCGGGGTTTCGGCTT  
CTGGCGTGTGACCGGCGGCTCTAGAGCCTCTGCTAACCATGTTTCATGCCTTCT  
TCTTTTCTACAGCTCCTGGGCAACGTGCTGGTTATTGTGCTGTCTCATCATT  
TGGCAAAGAATTAGCTTATAACTTCGTATAGCATAATTATACGAAGTTATAAC  
TAGTGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGATCCATCGCCACC  
ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTTCG  
AGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCG  
AGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGG  
CAAGCTGCCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTG  
CAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTC  
CGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGAC  
GGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTG  
AACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGG



GGCACAAGCTGGAGTACAACACAGCCACAACGTCTATATCATGGCCGA  
CAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAG  
GACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGC  
GACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCCAGTCCGCCC  
TGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGT  
GACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGC  
CGGCCGCGACTCTAGAGGATCATAATCAGCCATACCACATTTGTAGAGGTTTT  
ACTTGCTTTAAAAACCTCCACACCTCCCCCTGAACCTGAAACATAAAATGA  
ATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAG  
CAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTCACTGCATTCTAGTTGT  
GGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCTGAATTCATAAC  
TTCGTATAGCATACATTATACGAAGTTATAGGATCCGAAGTTCCTATTCTCTAG  
AAAGTATAGGAACTTCCCCATGGGTAGAGGTTCTCATCATCACCATCACCATA  
TGCCAGCGCGAACAGCTCCAGCCCGAGTGCCTGCGCTTGCTCCCCGGCAGG  
CTCGCTCCCGGACCATGTCCGCAGGCGGCTCAAAGACTTGGAAGAGATGGC  
TTAACAGAAAAGGAGTGTGTGAGGGAGAAATTAACTTACTGCATGAATTCCT  
GCAAACAGAAATAAAAAAGCCAGTTGTGTGACTTGGAACCAAATTACATAAAG  
AGGAATTATCTGAGGAAGGCTACCTGGCTAAAGTCAAGTCCCTCTTAAATAAG  
GATTTGTCCTTGAGAACGGAACACACACTCTCACTCAAAAAGCCAACGGTTG  
TCCCGCCAACGGGAGCCGGCCAACCTGGAGAGCAGAAATGGCAGACTCAAA  
TAGATCCCCAAGATCCAGGCCCAAGCCTCGGGGACCCAGGAGAAGCAAGTC  
GGACAGTGACACCCTTTTTGAACTTCACCTAGTTCCGTGGCTACGAGGAGAA  
CCACCAGGCAGACCACCATCACGGCTCACTTCACGAAGGGCCCCACTAAACG  
GAAACCCAAGGAAGAGTCGGAAGAGGGGAACTCGGCTGAGTCGGCTGCAGA  
GGAGAGAGACCAGGATAAGAAACGCAGAGTTGTAGACACAGAGAGTGGTGC  
TGCAGCTGCTGTGGAGAACTGGAAGAGGTAACAGCGGGAACCCAGCTGGGT  
CCGGAAGAGCCATGTGAACAGGAAGATGACAACAGGAGTCTTCGACGTCACA  
CCAGAGAGCTATCATTGAGGCGGAAATCAAAGGAGGATCCAGACAGAGAAGC  
AAGACCGGAACTCACTTGACGAGGACGAGGACGGAAAAAAGGATAAAAG  
AAGTTCCAGACCCAGGAGCCAGCCCAGAGATCCAGCTGCCAAACGGAGACC  
CAAGGAAGCAGAGCCAGAGCAGGTAGCTCCAGAGACTCCCGAGGACAGAGA  
CGAGGATGAGAGGGAGGAGAAGAGACGAAAAACGACACGTAAAAAACTGGA  
GTCACACACCGTTCCCGTTCAGAGCAGATCGGAGAGAAAAGCCGCTCAAAGC

AAAAGTGTGATCCCGAAGATCAACTCACCAAAGTGCCCCGAGTGTGGCCAGC  
ACCTAGACGACCCTAACCTGAAGTACCAGCAGCACCTGAGGATGCTGTGGA  
TGAACCCCAGATGTTGACCAGTGAGAACTGTCCATCTACGACTCCACCTCGA  
CCTGGTTTGATACTTATGAAGATTCTCCCATGCATAGGTTCACTTCCTTCAGTG  
TGTA CTGCAGTCGCGGGCACCTGTGTCCTGTGACACCGGTCTCATTGAGAAG  
AATGTAGAGCTCTACTTTTCTGGGTGTGCCAAAGCAATTCATGACGAGAATCC  
ATCTATGGAAGGTGGTATTAATGGCAAAAACCTCGGGCCAATCAATCAGTGGT  
GGCTCAGTGGCTTTGATGGTGGCGAGAAGGTGCTCATTGGCTTCTCCACTGCA  
TTTGCTGAATACATTTTGATGGAGCCCAGCAAAGAGTATGAGCCAATATTTGG  
GCTGATGCAGGAGAAAATTTACATCAGCAAGATTGTTGTTGAGTTCCTGCAAA  
ACAATCCTGATGCTGTATATGAAGACCTGATCAATAAGATTGAGACCACTGTT  
CCTCCTTCTACCATTAATGTGAACCGGTTACAGAGGACTCCCTCTTACGCCA  
CGCCCAGTTTGTAGTGAGCCAGGTAGAGAGTTACGACGAAGCCAAGGACGAT  
GATGAGACCCCCATCTTCTTGTCTCCCTGTATGAGAGCCCTGATCCATTTGGCT  
GGTGTCTCCCTGGGACAGAGGCGAGCAACAAGGCGCGTCATGGGTGCTACCA  
AGGAGAAGGACAAAGCACCCACGAAAGCCACCACCACCAAGCTGGTCTATCA  
GATCTTTGACACTTTCTTCTCAGAGCAGATTGAGAAGTATGATAAGGAGGACA  
AGGAGAATGCCATGAAGCGCCGCGCTGTGGTGTCTGTGAGGTCTGTCAGCA  
GCCTGAGTGTGGGAAGTGCAAGGCGTGCAAAGATATGGTGAAGTTTGGTGGC  
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TGAAGGAGGCAGACGACGATGAAGAGGCTGATGATGATGTGTCAGAGATGCC  
ATCACCCAAAAAGCTGCATCAGGGGAAGAAGAAGAAGCAGAACAAGGACCG  
CATCTCCTGGCTTGGGCAGCCTATGAAGATTGAAGAGAATAGAACTTACTATC  
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ATGATCTGC

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## **Erklärung:**

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-

Fachbereiche zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „A Mouse Model for the Overexpression of Methyltransferases“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Nicholas Wagner befürworte.

Essen, den 30.11.2009

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Essen, den 30.11.2009

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